May 1991

Nitrogen Dioxide Reaction With Proteins: Evidence for Peptide Bond Cleavage at Lysine Residues

Darryl B. Hood
East Tennessee State University

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Nitrogen dioxide reaction with proteins: Evidence for peptide bond cleavage at lysine residues

Hood, Darryl Brice, Ph.D.
East Tennessee State University, 1991
NITROGEN DIOXIDE REACTION WITH PROTEINS:
EVIDENCE FOR PEPTIDE BOND CLEAVAGE AT LYSINE RESIDUES

A Dissertation
Presented to
the Faculty of the Department of Biochemistry
James H. Quillen College of Medicine
East Tennessee State University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy in Biomedical Sciences

by
Darryl B. Hood
May 1991
APPROVAL

This is to certify that the Graduate Committee of

DARYL B. HOOD

met on the

twenty-first day of August, 1990

The committee read and examined his dissertation, supervised his defense of it in an oral examination, and decided to recommend that his study be submitted to the Associate Vice-President for Research and Dean of the Graduate School, in partial fulfillment of the requirements for the degree Doctor of Philosophy in Biomedical Science.

Chair, Graduate Committee

Signed on behalf of the Graduate Council

Associate Vice-President for Research and Dean of the Graduate School
ABSTRACT

REACTION OF NITROGEN DIOXIDE WITH PROTEINS: EVIDENCE FOR PEPTIDE BOND CLEAVAGE AT LYSINE RESIDUES

by

Darryl B. Hood

Nitrogen dioxide (NO₂), an air pollutant produced by burning fossil fuels and a component of cigarette smoke, is thought to contribute to the pathogenesis of pulmonary diseases, such as emphysema. To gain information on the mechanism by which NO₂ damages the lung, in vitro exposures of α₁-proteinase inhibitor (α₁-PI), elastin, bovine serum albumin (BSA), human serum albumin (HSA) and synthetic poly-L-lysine were performed. A genetic deficiency of α₁-PI predisposes humans to emphysema and NO₂ has been hypothesized to damage α₁-PI, which would leave proteases such as human neutrophil elastase, (HNE) free to attack lung structural proteins. The ability of α₁-PI to inhibit HNE declined with exposure to 50% of the control value at molar ratios of NO₂:α₁-PI of 400:1 and greater. Exposure of α₁-PI to NO₂ resulted in a 50% loss of immunoreactivity with either monoclonal or polyclonal antibodies in an enzyme-linked immunosorbent assay at molar ratios of NO₂:α₁-PI of essentially 100:1 and greater. The mechanisms of these effects were investigated via ultraviolet-visible spectroscopy and amino acid analysis. The remaining target molecules were labelled by reductive methylation of amino groups with ³H-HCHO prior to treatment with NO₂ in aqueous solutions at physiological pH. Time course exposure of 5 mg ³H-insoluble bovine ligamentum nuchae elastin suspensions with up to 120 μmoles of NO₂ resulted in 90% solubilization of the label. Amino acid analysis of the soluble and insoluble fractions from these exposures confirmed that 80% of the ³H-dimethyllysine residues were in the soluble fraction. Since these results suggested a specific reactivity of NO₂ with lysine residues, 400 μg ³H-poly-L-lysine was exposed to 120 μmoles NO₂. Gel filtration chromatography revealed that the 50,000 M₀ ³H-poly-L-lysine had been degraded to small peptides of 1-3,000 M₀. Similar exposures were conducted using ³H-BSA and ³H-HSA, followed by gel filtration chromatography and SDS-PAGE with fluorography. The results suggest that NO₂ preferentially reacts with Lys-Lys or other specific sequences, resulting in peptide bond cleavage. Under the conditions used, 23% of the BSA tyrosine residues were nitratated and aggregates of HSA indicative of bityrosine cross-link formation were observed. These findings are the first indication that NO₂ can cause protein fragmentation and provide additional data on the potential of NO₂ to damage lung proteins, such as elastin.
DEDICATION

- To the Lord Almighty for giving me the strength and wisdom to persevere.
- To my loving and supportive parents, Dr. Calvin A. and Anna M. Hood. Through example, you have always impressed upon me the truly gratifying accomplishments in life, are worth working diligently for.
- To Karol (my future wife), words cannot explain the depth of our experiences during our matriculation through graduate school. My success was directly proportional to the level of support that you provided. I love you and am looking forward to the great experiences the future has in store for us.
- To Marius, in hopes that this may serve as a source of inspiration in the future. Son, the sky is the limit, you can accomplish anything in this world that you so desire, if you put your mind to it!
- To Dave and Lou, who through their never ending encouragement and support, molded me into a scientist.
- . . . I am the eminent expression of friendship . . . I inspire the musician to play noble sentiments and assist the chemist to convert ungenerous personalities into individuals of great worth . . . Lives once touched by me become tuned and are thereafter amiable, kindly, fraternal.
ACKNOWLEDGEMENTS

- I'd like to thank the members of my dissertation committee Drs. Johnson, Ernst-Fonberg, Robinson, DeLucia, and Skalko for believing in and supporting me for the duration.

- I'd like to thank the faculty, staff and graduate students of the Department of Biochemistry for a very fruitful, meaningful learning experience. I wish you all the best!

- Wei (Chung Lung) Chu, Robbie (Robo-Cop) Waites, Craig (Bork) Smith, and Bob Roudabush were inspirational to me and they will be rich in 10 years thanks to industry. On the other hand, Majid (Terrorist) Mehrpouyan and Margie (Margot Kidder) Tucker were also inspirational to me, however, we will be impoverished in 10 years.

- Raymonde Cox and Karen Ford's help will always be remembered.

- . . . To a few, I am the castle of dreams-ambitious, successful, hopeful dreams. To many, I am the poetic palace where human feeling is rhymed to celestial motives; to the great majority, I am the treasury of good fellowship.

- In fact, I am the college of friendship the university of brotherly love; the school for the better making of men.

. . . Ithaca, N.Y. 1906
# CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>APPROVAL</td>
<td>ii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>iv</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xii</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>xi</td>
</tr>
<tr>
<td><strong>CHAPTER</strong></td>
<td></td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Sources of Nitrogen Oxides (Nitrooxides)</td>
<td>3</td>
</tr>
<tr>
<td>Chemistry</td>
<td>9</td>
</tr>
<tr>
<td>The Toxicology of Nitrooxide Free-Radicals Present in the Gas Phase of Cigarette Smoke</td>
<td>10</td>
</tr>
<tr>
<td>The Inactivation of the Major Protease Inhibitor in Humans by Cigarette Smoke Free-Radicals</td>
<td>14</td>
</tr>
<tr>
<td>Nitrooxides and Emphysema</td>
<td>17</td>
</tr>
<tr>
<td>Elastin and the Connective Tissue Components of the Lung</td>
<td>18</td>
</tr>
<tr>
<td>Degradation of Connective Tissue Components by Oxidants <em>in vitro</em></td>
<td>20</td>
</tr>
<tr>
<td>Destruction of Lung Connective Tissues <em>in vivo</em> by Oxidants</td>
<td>21</td>
</tr>
<tr>
<td>Exposure to Nitrooxides Produces Emphysema-Like Lesions in Animals</td>
<td>22</td>
</tr>
<tr>
<td>Overview</td>
<td>24</td>
</tr>
<tr>
<td>2. MATERIALS AND METHODS</td>
<td>27</td>
</tr>
<tr>
<td>Purification of Alpha-1-Proteinase Inhibitor from Plasma</td>
<td>29</td>
</tr>
</tbody>
</table>

vi
<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrophoresis of α₁-Proteinase Inhibitor</td>
<td>31</td>
</tr>
<tr>
<td>Inhibitory Activity Assays</td>
<td>32</td>
</tr>
<tr>
<td>Labelling of Elastin</td>
<td>33</td>
</tr>
<tr>
<td>Labelling of Bovine Serum Albumin (BSA), Human Serum Albumin (HSA), and Poly-L-Lysine</td>
<td>34</td>
</tr>
<tr>
<td><em>In Vitro</em> Exposure of Proteins and Peptide to NO₂</td>
<td>35</td>
</tr>
<tr>
<td>Immunoenzyme Assays</td>
<td>38</td>
</tr>
<tr>
<td>Amino Acid Analysis</td>
<td>39</td>
</tr>
<tr>
<td>Electrophoresis and Fluorography of ³H-BSA &amp; ³H-HSA</td>
<td>41</td>
</tr>
<tr>
<td>Gel Filtration Chromatography</td>
<td>42</td>
</tr>
<tr>
<td>RESULTS</td>
<td>43</td>
</tr>
<tr>
<td>Purification of Alpha-1-Proteinase Inhibitor</td>
<td>43</td>
</tr>
<tr>
<td>Molar Stoichiometry</td>
<td>45</td>
</tr>
<tr>
<td>Effects of NO₂ on the Activity and Immunoreactivity of α₁PI</td>
<td>47</td>
</tr>
<tr>
<td>Exposure of ³H-Elastin</td>
<td>64</td>
</tr>
<tr>
<td>Exposure of ³H-Poly-L-Lysine</td>
<td>67</td>
</tr>
<tr>
<td>Exposure of BSA</td>
<td>70</td>
</tr>
<tr>
<td>Exposure of HSA</td>
<td>76</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>81</td>
</tr>
<tr>
<td>General Aspects of Direct Oxidative Damage to Lung Proteins</td>
<td>81</td>
</tr>
<tr>
<td>Modification of Tertiary Structure Following Exposure to NO₂</td>
<td>82</td>
</tr>
<tr>
<td>The Effects of NO₂ on Elastin</td>
<td>85</td>
</tr>
<tr>
<td>Protein Aggregation and Fragmentation as a Result of NO₂ Exposure</td>
<td>88</td>
</tr>
<tr>
<td>CHAPTER</td>
<td>Page</td>
</tr>
<tr>
<td>-----------------------</td>
<td>------</td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>95</td>
</tr>
<tr>
<td>APPENDIX</td>
<td>107</td>
</tr>
<tr>
<td>VITA</td>
<td>118</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Some of the Various Forms of the Nitrogen Oxides</td>
<td>5</td>
</tr>
<tr>
<td>2.</td>
<td>Purification of Human $\alpha$-1-PI</td>
<td>44</td>
</tr>
<tr>
<td>3.</td>
<td>Amino Acid Analysis of MSA Hydrolyzates of Native $\alpha_1$-PI, and of the Yellow, Insoluble, Precipitated Material Formed in the 1600:1 NO$_2$ Exposed $\alpha_1$-PI Sample</td>
<td>59</td>
</tr>
<tr>
<td>4.</td>
<td>Amino Acid Analysis of Control and NO$_2$ Exposed $\alpha_1$-PI</td>
<td>63</td>
</tr>
<tr>
<td>5.</td>
<td>$^3$H-Dimethyllysine Release from Control and NO$_2$ Exposed $^3$H-Elastin</td>
<td>65</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Interconversions of NO&lt;sub&gt;ω&lt;/sub&gt;</td>
<td>11</td>
</tr>
<tr>
<td>2.</td>
<td>Possible Physiological Consequences of the Nitroxide Free-Radical Components of Cigarette Smoke</td>
<td>16</td>
</tr>
<tr>
<td>3.</td>
<td>In Vitro NO&lt;sub&gt;2&lt;/sub&gt; Exposure System</td>
<td>36</td>
</tr>
<tr>
<td>4.</td>
<td>Titration of Proteases with α&lt;sub&gt;1&lt;/sub&gt;-PI</td>
<td>46</td>
</tr>
<tr>
<td>5.</td>
<td>The Effect of NO&lt;sub&gt;2&lt;/sub&gt; on the Inhibition of HNE by α&lt;sub&gt;1&lt;/sub&gt;-PI</td>
<td>48</td>
</tr>
<tr>
<td>6.</td>
<td>Electrophoresis of α&lt;sub&gt;1&lt;/sub&gt;-PI:HNE Complexes</td>
<td>49</td>
</tr>
<tr>
<td>7.</td>
<td>Immunoenzyme Assay of Control and NO&lt;sub&gt;2&lt;/sub&gt; Exposed α&lt;sub&gt;1&lt;/sub&gt;-PI (1 nmol/ml)</td>
<td>51</td>
</tr>
<tr>
<td>8.</td>
<td>NO&lt;sub&gt;2&lt;/sub&gt; Exposed α&lt;sub&gt;1&lt;/sub&gt;-PI Immunoreactivity</td>
<td>52</td>
</tr>
<tr>
<td>9.</td>
<td>Immunoenzyme Assay of Control and NO&lt;sub&gt;2&lt;/sub&gt; Exposed α&lt;sub&gt;1&lt;/sub&gt;-PI (4 nmole/ml)</td>
<td>54</td>
</tr>
<tr>
<td>10.</td>
<td>Statistical Analysis of Control and NO&lt;sub&gt;2&lt;/sub&gt; Exposed α&lt;sub&gt;1&lt;/sub&gt;-PI Immunoreactivity</td>
<td>55</td>
</tr>
<tr>
<td>11.</td>
<td>Total Protein determination on Control and NO&lt;sub&gt;2&lt;/sub&gt; Exposed α&lt;sub&gt;1&lt;/sub&gt;-PI by the OPA Method</td>
<td>56</td>
</tr>
<tr>
<td>12.</td>
<td>SDS-PAGE Analysis of the Yellow Insoluble Material Formed from NO&lt;sub&gt;2&lt;/sub&gt; Exposure of α&lt;sub&gt;1&lt;/sub&gt;-PI</td>
<td>58</td>
</tr>
<tr>
<td>13. A</td>
<td>UV-Visible Absorption Spectra for Native and NO&lt;sub&gt;2&lt;/sub&gt; Exposed α&lt;sub&gt;1&lt;/sub&gt;-PI (4 nmole/ml) and 72 hr. Nitrogen Bubbled Control α&lt;sub&gt;1&lt;/sub&gt;-PI (4 nmol/ml)</td>
<td>61</td>
</tr>
<tr>
<td>B)</td>
<td>UV-Visible Absorption Spectra of 1mg/ml solutions of HSA</td>
<td>62</td>
</tr>
<tr>
<td>14.</td>
<td>&lt;sup&gt;3&lt;/sup&gt;H-Dimethyllysine Release from NO&lt;sub&gt;2&lt;/sub&gt; Exposed &lt;sup&gt;3&lt;/sup&gt;H-Elastin</td>
<td>66</td>
</tr>
<tr>
<td>15. A</td>
<td>Gel Filtration Analysis of Control &lt;sup&gt;3&lt;/sup&gt;H-Poly-L-Lysine</td>
<td>68</td>
</tr>
<tr>
<td>B)</td>
<td>Gel Filtration Analysis of NO&lt;sub&gt;2&lt;/sub&gt; Exposed &lt;sup&gt;3&lt;/sup&gt;H-Poly-L-Lysine</td>
<td>69</td>
</tr>
<tr>
<td>FIGURE</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>16. A) Gel Filtration Analysis of Control (^3\text{H-BSA})</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>B) Gel Filtration Analysis of (\text{NO}_2) Exposed (^3\text{H-BSA})</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>17. A) SDS-PAGE Analysis of (^3\text{H-BSA}) after Exposure to (\text{NO}_2)</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>B) Fluorography</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>18. Fluorography Following 10% SDS-PAGE Analysis of (^3\text{H-HSA}) after Exposure to (\text{NO}_2)</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>19. A) Time Course SDS-PAGE Analysis of the Yellow, Insoluble Material Formed from (\text{NO}_2) Exposure of (^3\text{H-HSA})</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>B) Fluorography</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>20. Schematic of Proposed Cleavage of HSA at Lysine-Lysine Sequences by (\text{NO}_2) to Generate Peptides With and Without Tyrosine Residues</td>
<td>92</td>
<td></td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

\(\alpha_{1}\)-PI  alpha-1-proteinase inhibitor

ppm  parts per million

HNE  human neutrophil elastase

PPE  porcine pancreatic elastase

PPT  porcine pancreatic trypsin

SDS  sodium dodecyl sulfate

PAGE  polyacrylamide gel electrophoresis

TCA  trichloroacetic acid

DMSO  dimethyl sulfoxide

pNA  paranitroanilide

Bz  benzoyl

MES  morpholino-ethane sulfonic acid

Tris  tris(hydroxymethyl)aminomethane

HEPES  N-2-Hydroxy-ethylpiperazine-N’-2-ethanesulfonic acid

NEDA  N-1-Naphthylethlenediamine

MSA  methanesulfonic acid

PITC  phenylisothiocyanate

DTT  dithiothreitol

DTNB  5,5′-dithiobis-(2-nitrobenzoic acid)

GSH  glutathione

BSA  bovine serum albumin

HSA  human serum albumin

cpm  counts per minute

DEAE  diethylaminoethyl
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC</td>
<td>high pressure liquid chromatography</td>
</tr>
<tr>
<td>EPA</td>
<td>environmental protection agency</td>
</tr>
<tr>
<td>PBN</td>
<td>phenyl-tert-butyl nitrone</td>
</tr>
<tr>
<td>EIC</td>
<td>elastase inhibitory capacity</td>
</tr>
<tr>
<td>CBS</td>
<td>cibacron blue sepharose</td>
</tr>
<tr>
<td>TBS</td>
<td>tris buffered saline</td>
</tr>
<tr>
<td>TEA</td>
<td>triethylamine</td>
</tr>
<tr>
<td>dH₂O</td>
<td>distilled water</td>
</tr>
</tbody>
</table>
Chapter 1
INTRODUCTION

Many people are becoming increasingly concerned about the state of the environment. Presently, environmental issues are being politicized in an effort to preserve for future generations a sanctuary that will be free of excessive pollution, including the undesirable health effects associated with air pollution. As a part of society, the scientific community has attempted to address some of the concerns relative to the risks to health associated with living in polluted environments. Under this umbrella there is an area of research that is directed at determining whether environmental pollutants such as ozone (O₃), and nitrogen dioxide (NO₂), alter the structure and function of lung proteins.

Various metropolitan areas have been identified by the United States Environmental Protection Agency (EPA) as having excessively high ambient concentrations of nitrogen dioxide, one of the major volatile air pollutants. The primary source of this ambient nitrogen dioxide originates from fossil fuel combustion in automobiles, so that automobile emissions are considered to be a major contributing factor to air pollution in these areas. Of the many health effects associated with air pollution, or more specifically, components of photochemical smog, lung
respiratory diseases have received the most attention. Since the lung represents the greatest point of exposure to the outside environment, it is not surprising that evidence has accumulated suggesting that inhalation of these environmental pollutants may have a deleterious effect on lung structure and function.

Presently, there is also a growing concern as to the health effects associated with tobacco smoke in indoor environments. Mainstream cigarette smoke is defined as that portion inhaled by the smoker, whereas "sidestream" smoke includes exhaled smoke as well as smoke that has not been inhaled. Both of these fractions of tobacco smoke contain gaseous nitrogen dioxide itself and related reactive nitrogen oxides. Cigarette smoking has been recognized as a primary risk factor for the development of emphysema, one of several respiratory diseases. Since nitrogen dioxide and its related oxides are present in the seemingly dependent variable related to the development of emphysema, its increasing presence in the atmosphere primarily due to increasing automobile emissions has attracted a considerable amount of attention. This interest stems from the possibility that nitrogen dioxide may play a role in damaging the lung and/or components of the lung when breathed over an extended period of time. Nitrogen dioxide, which is a component of cigarette smoke, and the burning of fossil fuels, causing outdoor air pollution, may be the
causative agent in the development of lung diseases such as emphysema.

When considering the health effects associated with exposure to nitrogen dioxide and its related oxides in terms of the development of emphysema, we must initially consider the major sources and the chemistry of the atmospheric nitrogen oxides. Consideration must also be given to the nitrogen oxide content of mainstream cigarette smoke and the toxicology of these nitrogen oxide species that are present in the gas phase of cigarette smoke. The effect of cigarette smoke on a protein that is instrumental in protecting the lung from proteolytic attack will also be considered. Animal model correlates which suggest a possible relationship between exposure to nitrogen dioxide and the development of emphysema will be considered. Finally, the structure of the lung itself, in terms of its connective tissue components and the degradation of these components in vivo and in vitro by oxidants will be considered.

Sources of Nitrogen Oxides (Nitroxides)

Three of the major nitrogen-containing compounds in the atmosphere are nitrous oxide (N₂O), nitric oxide (NO), and nitrogen dioxide (NO₂). These gaseous compounds arise in part from biological action and organic decomposition in the soil and ocean (Lee, 1980). It is a generally accepted practice to designate the many atmospheric forms of the
nitrogen oxides as NO\textsubscript{x} (nitroxides). The primary by-products of one electron reductions of the nitrogen oxides are nitrite (NO\textsubscript{2}\textsuperscript{-}), and nitrate (NO\textsubscript{3}\textsuperscript{-}), which contain unpaired electrons in their outer orbits. From a toxicological viewpoint, NO\textsubscript{2} is viewed as the dominant urban atmospheric pollutant, because NO and NO\textsubscript{2} are the major nitrogenous products of fossil fuel combustion (Fishbein, 1976). In addition to gaseous NO\textsubscript{x}, nitrate aerosols are produced in the atmosphere by the oxidation of nitrogen oxides generated during combustion of fossil fuels (Vostal et al. 1985). The EPA delineates various components of particulate air pollution based on whether it is a gas, an inhalable particulate (aerosol) or a particulate, incapable of being inhaled. In a study of metropolitan areas in Michigan, Colorado, and suburban areas in Virginia, Louisiana, and Delaware, it was found that the inhalable particulates (aerosols) formed two-thirds to three-quarters of the total inhalable particulate load (Vostal et al. 1985). It was concluded from this study that more research was needed on aerosols, if the undesirable health effects associated with inhaled particulates were to be prevented. It is common to use NO\textsubscript{2} and NO\textsubscript{x} interchangeably when the biomedical implications of air pollution are discussed (Morrow, 1984). Table 1 shows various forms of the nitrogen oxides that are of interest when the possible health effects of air pollution are considered.
Table 1. Some of the Various Forms of the Nitrogen Oxides.

<table>
<thead>
<tr>
<th>Form</th>
<th>Name</th>
<th>Form</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>N₂O</td>
<td>Nitrous oxide</td>
<td>HNO₂</td>
<td>Nitrous Acid</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
<td>HNO₃</td>
<td>Nitric Acid</td>
</tr>
<tr>
<td>NO₂</td>
<td>Nitrogen dioxide</td>
<td>N₂O₄</td>
<td>Dinitrogen tetroxide</td>
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<td></td>
<td>(dimeric NO₂)</td>
<td></td>
<td>(dimeric NO₂)</td>
</tr>
<tr>
<td>N₂O₃</td>
<td>Dinitrogen pentoxide</td>
<td>NO₃</td>
<td>Nitrogen trioxide</td>
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<td>(dimeric NO₂)</td>
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<td></td>
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<tr>
<td></td>
<td></td>
<td>N₂O₂</td>
<td>Nitrogen peroxide</td>
</tr>
</tbody>
</table>

The major biological source of NOₓ is anaerobic bacterial reduction of NO₃ to NO₂ in the soil. The resulting estimated NOₓ as NOₓ⁻ in soil is converted to nitrous acid (HNO₂), which decomposes to yield NO and NO₂ (Nelson et al. 1970).

Nitrous oxide (N₂O) is the most abundant atmospheric nitrogen oxide compound and has a mean concentration of 0.25 ppm in clean air and 1 ppm in polluted air (Lee, 1980). The major source of N₂O is from nitrogenous compounds in the soil, with an estimated 5.92 x 10⁸ tons/year being released into the atmosphere as a result of anaerobic denitrification (Fishbein, 1976). Nitrous oxide is considered to be a very stable and chemically inert gas which essentially undergoes no chemical conversions in the atmosphere. Its mean
residence time in the atmosphere is estimated to be four years (Fishbein, 1976).

The origin of atmospheric nitric oxide (NO) and nitrogen dioxide (NO₂) is from the oxidation of organically bound nitrogen present in fossil fuels and from the oxidation of molecular nitrogen in the atmosphere (Irvin, 1986). Among the nitrooxides, NO₂ plays the pivotal role in atmospheric reactions producing photochemical oxidants (Schwartz, 1986). Although where NO is the primary nitrooxide in automobile emissions, it is oxidized to the much more reactive NO₂ within several hours, and this photochemical process is primarily responsible for daily variations in nitrooxide concentrations in areas such as Los Angeles (Atkinson & Lloyd, 1984). Concentrations of nitrooxides peaking in association with rush hour traffic can be observed in certain urban centers and the local concentrations associated with human activity can be 25 to 500 times larger than the natural background (Berry et al. 1971). Occasionally, in scattered areas of the United States, peak levels of 0.4 - 5.0 ppm have been observed and concentrations in excess of 0.14 ppm have been shown to be quite common (EPA-600/8-82-026, 1982). The national ambient air quality standard for nitrogen dioxide is 0.05 ppm (Lee, 1980), and its residence time in the atmosphere has been estimated to be approximately two months (Wilson et al. 1980). Overall, when considering total anthropogenic
nitrooxide emissions, which are from electric power plants, stationary fuel combustion sources, and automobile emissions, the latter source has comprised approximately 45% of the total (Wilson et al. 1980).

Emissions from aircraft represents another polluting source of nitrogen oxides. The aircraft emissions (carbon monoxide, hydrocarbons, nitrogen oxides, and particulate matter) in the United States represent 3.3% of all emission sources (Hendel, 1973). Excessive pollution can occur in localized areas around airports. For example, in the vicinity of Los Angeles Airport and Chicago O’Hare Airport 32,660 and 29,540 tons/year of pollutants are released respectively. An estimated 16,000 tons of nitrogen oxides were released by all civil aircraft in the U.S. in 1967 (Hendel, 1973). Larger engines and higher temperatures which will be encountered in the futuristic, high tech aircraft engines, can be expected to make nitrooxide emissions from aircraft an even greater problem.

Within the last decade, a considerable amount of attention has been given to the question concerning the health risks associated with exposure to sidestream tobacco smoke. Simply put, is the non-smoker at risk while inhaling secondary smoke generated from a burning cigarette in indoor environments? Several recent studies have attempted to address this issue by initially identifying the chemical compounds and more importantly for the purposes of this
discussion, determination of the primary inorganic gases present in sidestream smoke. Some of the sidestream yields of nitrogen oxides (μg/ cigarette) were found to be on the order of 0.1 - 3.5 (Guerin et al. 1987); 2.2 - 2.6 (Eatough et al. 1989); 1.4 - 2.0 (Heavner et al. 1986); 5.3 - 8.5 (Klus & Begutter, 1987).

In addition to the concerns regarding nitrooxides that are present in sidestream smoke in indoor environments, other sources of nitrooxides in indoor environments exist. A popular concept emerging these days is that of the sick building syndrome. New buildings containing large amounts of synthetic components tend to give off nitrogenous and volatile organic compounds and others tend to "off-gas" at a slow pace for any number of years (Robertson, 1989). Office equipment, supplies, duplicators, copiers, furnishings, carpets, draperies have been found to be the major sources and nitrooxide vapors have been identified along with other gases (Robertson, 1989). Nitrooxides have also been associated with coal fires, kerosene heaters, wood fires, and gas stoves (Robertson et al. 1989).

With respect to cigarette smoke in indoor environments, the research is relatively new and efforts are primarily directed at identifying markers that can then be used to estimate the exposure of the non-smoker to sidestream smoke in indoor environments. At the present, long term estimates concerning the risk assessment of disease are being made in
an attempt to attract researchers to this very interesting and relevant area of work.

**Chemistry**

Once NO is released into the atmosphere, it is converted to NO$_2$ by a rapid oxidation process that is not completely understood (Morrow, 1984). One of the two favored explanations involves the reaction of NO with oxygen atoms. The source of oxygen atoms in the lower atmosphere is the photolysis of NO$_2$; NO$_2$ + hv $\rightarrow$ NO + O, which is also the initiating reaction for almost all photochemical smog reactions (Berry & Lehman, 1971). The other predominant process is the reaction of ozone (O$_3$) with NO to give NO$_2$ + O$_2$. The source of ozone is believed to be the downward diffusion from the stratosphere (Fishbein, 1976).

The conversion of NO to NO$_2$ in urban areas (where it is essentially complete) is very difficult to explain. As reviewed by Berry & Lehman (1971), the available amount of stratospherically generated ozone is apparently insufficient to account for the levels of NO$_2$ in urban air. The initial amount of NO$_2$ is believed to be formed in exhaust pipes and furnace stacks via the thermal oxidation of NO with molecular oxygen in a thermolecular process. Berry and Lehman (1971) summarized the reaction of NO$_2$ in a typical urban atmosphere such that the net result is the gradual consumption of NO and the production of oxygen atoms that can then initiate the reactions of photochemical smog. The
nitrogen is depleted by the reaction of NO$_2$ with hydrocarbon radicals produced by chain reactions initiated by oxygen atoms.

The chemistry of the nitrogen oxides is somewhat complicated and at present is not fully understood. Figure 1 shows some of the reactions of the environmental nitrogen oxides in terms of their interconversions.

The Toxicology of Nitrooxide Free-Radicals Present in the Gas Phase of Cigarette Smoke

The gas phase free-radicals that are present in cigarette smoke are very unstable and low in concentration (Church & Pryor, 1984). Therefore, attempts have been made to study these free-radicals using the electron spin resonance (ESR) technique (Pryor et al. 1976). The technique involves "trapping" unstable radicals with a compound such as phenyl-tert-butyl nitrene (PBN) to give a radical that is more stable than the radical that was trapped, allowing detection. Initial applications of this technique by Bluhm et al. (1971) to cigarette smoke suggested that nitrooxide radicals may have been formed but the information concerning the nature of the radicals that were trapped was sketchy.

Church and Pryor (1984) tested a variety of protocols for spin trapping the gas phase radicals from cigarette smoke. The results from these spin trapping studies
Figure 1. Interconversions of NO\textsubscript{x}. This schematic shows some of the reactions and interconversions of the nitrogen oxides. Where equilibria are shown, the length of the arrow approximates the favored reaction. (Based on Guidotti, 1978)
suggested that a vinyl nitroxide free-radical species was produced.

This was a first in terms of applying this type of methodology to an environmental problem. In this study a smoke stream was bubbled through a solution of PBN in carbon tetrachloride, or benzene. An unusual feature of the trapped ESR gas phase free-radicals observed in this study was that they had amazingly long lifetimes in the gas phase, which was inconsistent with the known chemical stabilities of oxygen and carbon centered radicals in cigarette smoke (Pryor et al. 1976). The authors concluded that the small organic free-radicals that were trapped from the gas phase smoke could not be those that were produced during the well known combustion process. As reviewed by Church and Pryor (1984), the radicals formed from the combustion of tobacco are short lived radicals that undergo termination reactions while flowing down the tobacco column in the cigarette; therefore, these radicals do not reach the spin trap solution, which would indicate that they would be incapable of reaching the lung of smokers also.

These observations led to a steady-state hypothesis for radical formation in cigarette smoke, which holds that radicals are continuously being formed and destroyed as the result of gas phase radical reactions. This scenario involves the interactions of nitroxides with other constituents in smoke. As mentioned earlier, fresh
cigarette smoke is known to contain very little if any NO₂, it contains primarily nitric oxide as the principal nitroxide. The NO levels in smoke are extremely high relative to the levels in smog, 300-500 ppm (Guerin, 1980). Nitric oxide is relatively unreactive with most organic species, but it undergoes slow oxidation to the much more reactive NO₂ species. With these facts at hand, Church and Pryor felt that nitrogen dioxide could very possibly undergo facile reactions with many of the species in cigarette smoke. The compound isoprene is a reactive diene that occurs in high levels in cigarette smoke (Guerin, 1980), so it was reacted with NO and this reaction mixture was shown to generate carbon-centered radicals (Church & Pryor, 1985) and was thus recognized as a synthetic model of cigarette smoke.

The results revealed that the spin trap adducts formed from the synthetic NO/isoprene/air mixture were virtually identical to the actual cigarette smoke spin adducts, which indicated that the major spin adduct in the synthetic model (NO, isoprene, air) was an alkoxy radical. The data from the NO/isoprene/air synthetic model was viewed as very encouraging and established the possibility of a pathway involving a reactive nitroxide species and a reactive organic compound which could contribute to free-radical production (Pryor et al. 1984).
The Inactivation of the Major Protease Inhibitor
in Humans by Cigarette Smoke Free-Radicals

α₁-Proteinase inhibitor (α₁-PI) accounts for approximately 90% of the elastinolytic inhibitory activity in the bronchoalveolar lavage fluid of normal individuals (Snider, 1981). The classic report by Laurell and Eriksson (1963) found that individuals with a genetic deficiency of α₁-PI were predisposed for the development of emphysema. These findings led to the protease-protease inhibitor balance theory whereby lung connective tissue components such as alveolar elastin, which are normally protected from elastolytic proteinases, such as neutrophil elastase, are attacked by elastases due to an overproduction of these enzymes or a failure in the inhibitors. A mechanism for the development of pulmonary emphysema was suggested wherein α₁-PI the major regulator of neutrophil elastase in the lower respiratory tract was inactivated by oxidants, resulting in a lung-localized deficiency of active α₁-PI (Janoff et al. 1987). This finding was critically important in terms of understanding the pathology of emphysema, since the genetic deficiency accounts for only 1% of patients suffering from chronic obstructive pulmonary lung diseases (Snider, 1981). Conversely, cigarette smoking is considered to be the major risk factor in the greater majority of patients suffering from emphysema (Auerbach et al. 1972).
Cigarette smoking has been shown to decrease the elastase inhibitory capacity (EIC) of pulmonary lavage fluids in humans (Carp et al. 1982; Gadek et al. 1979) and in the serum of rats (Janoff et al. 1979). The loss of EIC has been shown not to result from decreased levels of α₁-PI but rather from the formation of an inactive form of the inhibitor which contains oxidized methionine residues (Carp et al. 1982). One methionine residue in α₁-PI is at the active site and it is essential for the elastase binding ability of the protein (Johnson & Travis, 1978).

The role of cigarette smoke in mediating the oxidation of α₁-PI is not fully understood. Figure 2 shows plausible pathways by which this oxidation could occur and also shows, more generally, potential pathways by which oxidant damage could occur to other physiological target molecules.

Cigarette smoke is regarded as a complex chemical system, and there are several pathways for the many reactive species to interact with one another and with biopolymers in a smoker's lung (Pryor et al. 1982). Evidence is mounting which suggest that free-radical processes may play a significant role in cigarette smoke toxicology. Reaction a (Hocking et al. 1979) shows that pulmonary alveolar macrophages are activated by components of cigarette smoke; this process produces superoxide ion and hydrogen peroxide, and these reactive oxygen species can directly cause biological damage. The schematic also shows nitric oxide
Figure 2. Possible Physiological Consequences of the Nitroxide Free-Radical Components of Cigarette Smoke.
(NO) and various organic molecules (such as isoprene) that are produced in the smoke stream in reaction b (Church & Pryor, 1984). Nitric oxide is very unreactive, but it undergoes oxidation to form nitrogen dioxide (reaction c). NO₂ can react with hydrogen peroxide (reaction d) to produce a species that has been shown to inactivate α₁-PI (Dooley & Pryor, 1982). These species are thought to include superoxide and hydroxyl radicals based on the protection that was observed with free-radical scavengers. Also, NO₂ could react with other gaseous components of cigarette smoke (isoprene) to form alkyl radicals (reaction h), which can peroxidize (reaction i), and undergo deoxygenation with NO or NO₂ to form alkoxy radicals (reaction j). Alkoxy radicals are known to be extremely reactive and they are thought to cause damage to the lung (reaction k). Also NO and NO₂ (reaction f) (Church & Pryor, 1984) oxidize thiols to disulfides, which could oxidize glutathione in the lung, which would alter the thiol/disulfide redox balance, causing extra-pulmonary changes in blood chemistry (reaction g). Reaction e is the primary focus of the present study and involves a direct oxidative effect of NO₂ on elastin, and other proteins, such as the serum albumins.

**Nitroxides and Emphysema**

The mounting evidence which supports a causal role for oxidants in the pathogenesis of emphysema is based on experiments demonstrating that: (1) oxidants are capable of
degrading connective tissue \textit{in vitro} (Vemkatasubramanian & Joseph, 1983), (2) lung connective tissue is degraded in animals exposed to oxidants (Kleirnerman, 1979), and (3) animals exposed to oxidant gases develop an emphysema-like lesion several weeks after exposure (Riley & Kerr, 1985).

\textbf{Elastin and the Connective Tissue Components of the Lung}

The alveolar capillary membrane represents the largest bulk of the lung parenchyma (matrix) and it contains the major components in the pericapillary structure (Turino, 1985). The structural components of the intercellular substance of the lung are collagen, which constitutes 60-70\% of the interstitium (Hance & Crystal, 1976); elastin, 25-30\% (Chrzanowski et al. 1980); glycosaminoglycans, less than 1\% (Schmid et al. 1982); and fibronectin, 0.5\% (Bray, 1978).

Elastin is found deposited in almost all organs of the body, but is present in greater proportions in the lung, blood vessels, and skin (Turino, 1985) where it is the functional protein component of the elastic fiber.

The matrix of the lung serves not only as a framework for cell growth and orientation but it contributes to the mechanical characteristics of the lung parenchyma itself, and therefore has been recognized as an important structural element in the pressure-volume characteristics of the whole lung (Chrzanowski et al. 1980). When it was shown experimentally that altering certain structural elements in the matrix at the molecular level could alter the entire
pressure-volume behavior of the lung (Johanson & Pierce, 1972), a new light was cast on this area of research. One enzyme (an elastase), which had previously been recognized as a pancreatic constituent (Balo & Banga, 1949), was shown to disturb the recoil characteristics of the lung. By the predominant degradation of elastin in the lung parenchyma as opposed to collagen or glycosaminoglycans, an increase in the stretch of tissue was observed as well as loss of elastic support for airway patency in inspiration and expiration (Turino, 1985). Other proteases, such as collagenases (Johanson & Pierce, 1972; Snider et al. 1977) and hyaluronidases (Turino et al. 1968), did not display this characteristic action. These studies served as a biological confirmation to concepts that were first presented in the mid-fifties (Setnikar, 1955) and provided insights into the generalized structural importance of various matrix components. Attention was also given to the fact that these components were also vulnerable to proteolytic enzymes in the in vivo environment, such as the elastases from neutrophils (Janoff & Scherer, 1968) and macrophages (Rodriguez et al. 1977), which are capable of degrading elastin. The elastases were thought to be the primary mediators of matrix injury when it was shown that they could produce emphysema-like lesions in rats (Blackwood et al. 1973). This observation, together with the discovery of the relationships of $\alpha_1$-PI genetic deficiency and
emphysema by Laurell and Ericksson (1963), led to the strengthening of the protease-protease inhibitor imbalance hypothesis.

Degradation of Connective Tissue Components by Oxidants in vitro

Oxidants (any species with an unpaired electron in its outer orbit) have been shown to be capable of non-enzymatically degrading components of connective tissue in vitro (Riley & Kerr, 1985). Several in vitro studies in recent years have contributed to the understanding of the interaction of reactive oxygen species and connective tissue components. Collagen in solution was degraded by free-radicals, as evidenced by its failure to gel normally at 37°C (Greenwald & Moy, 1979). Soluble collagen molecules are cleaved into small peptides when incubated with a system producing superoxide anions (Monboisse et al. 1983) or hydroxyl radicals produced by Fenton's reagent (which is essentially hydrogen peroxide that is catalytically decomposed by transition metals such as Fe²⁺ to produce hydroxyl radicals) (Durham & Brock, 1986). Ozone also degrades soluble collagen, and this reaction is inhibited by free-radical scavengers (Curran et al. 1984). Intermittent exposure of collagen to oxidants enhanced its susceptibility to degradation by proteases (Curran et al. 1984). Hydrogen peroxide pretreatment of partially purified preparations of glomerular basement membrane glycoprotein, and fibronectin,
increases their susceptibility to proteolysis by several purified enzymes (Fligiel et al. 1984; Vartio, 1989). The \textit{in vivo} significance of these studies, in terms of how these forms of injury might relate to human disease, is unclear because the overall challenge of free-radical exposure \textit{in vitro} was more likely greater than the challenge \textit{in vivo}. However, these studies do support the contention that connective tissue components may be degraded \textit{in vivo}, provided a sufficiently high level of free-radical oxidants reached the extracellular matrix tissues (Riley & Kerr, 1985).

\textit{Destruction of Lung Connective Tissue \textit{in vivo} by Oxidants}

Previous studies have suggested that lung connective tissue is degraded in animals that have been exposed to oxidant gases (Kleinerman, 1979). The majority of these studies have focused on collagen degradation rather than elastin degradation. This seems ironic since the hallmark of emphysema is the degradation of elastin.

Hamsters exposed to 30 ppm NO$_2$ for 10 days have decreased collagen and elastin contents in their lungs (Kleinerman & Cowdrey, 1968). Lung collagen is reduced in guinea pigs exposed to 2 mg/m$^3$ NO$_2$ for 6 months (Drozdz et al. 1977). Based on turnover studies after intermittent exposures with O$_3$ (Richmond & D’Aoust, 1976), it seems that a sustained exposure to oxidant gases is necessary to cause lung collagen and elastin degradation. Models involving the
intratracheal instillation of enzyme systems which generate reactive oxygen species transiently decrease lung collagen content 3 days after exposure (Phan et al. 1982). In a similar study, diminished staining for connective tissue components in alveolar walls was noted 3 days following intratracheal instillation of the enzyme system (Sandbloom et al. 1982). Hydroxyproline is an amino acid marker for lung collagen degradation and increased levels of this marker are found in the lung lavage of rats exposed to hyperoxia (Riley et al. 1983). The overall mechanism of destruction of lung connective tissue during acute oxidant lung injury is unknown; however, proteolysis is thought to be involved. At present it remains unclear as to whether direct damage to connective tissue by oxidants occurs in vivo.

**Exposure to Nitroxides Produces Emphysema-Like Lesions in Animals**

As with most toxicological studies (Morrow, 1984), animal exposures to NO₂ have led to the development of animal models for emphysema (Freeman et al. 1968). Laboratory animals, such as monkeys (Ehrlich & Fenters, 1973), dogs (Lewis et al. 1973), rabbits (Haydon et al. 1963), guinea pigs (Drozdz et al. 1977; Gross et al. 1968), and rats (Freeman et al. 1968; Freeman & Hayden, 1964; Freeman et al. 1969) have been exposed to oxidants, such as NO₂, over a period of time until distinct morphological,
focal signs of emphysema resulted. This condition in laboratory animals is known as NO₂ induced "experimental emphysema" (Glasgow et al. 1987) and is characterized by permanent enlargement of the alveolar airspaces and destruction of the elastin-rich alveoli (Snider et al. 1985), which occurs after a latent period of exposure to NO₂ (Glasgow et al. 1987). This NO₂ induced lesion resembles the early centrilobular emphysema-like lesion in humans. Centrilobular emphysema in humans is primarily associated with cigarette smoking, where the alveoli adjacent to respiratory bronchioles are selectively destroyed, but the more distal alveolar tissue is spared (Evans et al. 1989).

In contrast, other animal models of the disease have been developed by intratracheal instillation of porcine pancreatic elastase. Elastase-induced emphysema is histologically similar to human panlobular emphysema (Kaplan et al. 1973). Panlobular emphysema is characterized histologically by the destruction of all components of the acinus. Early onset, familial emphysema occurs in individuals with a genetic deficiency of alpha-1-proteinase inhibitor, these individuals develop panlobular emphysema rather than centrilobular emphysema (Evans et al. 1989).

NO₂ induced experimental emphysema differs from elastase induced emphysema in that the NO₂ induced lesion develops after a latent period of exposure and does not progressively worsen with time, where as the intratracheal elastase
induced lesion develops rapidly and is very progressive in its pathology (Glasgow et al. 1987). These differences suggest that the two models of experimental emphysema are mechanistically different. The results of the in vivo studies of Glasgow and colleagues led them to conclude that the NO₂ induced lesion may more closely mimic the development of human emphysema in smokers.

**Overview**

Based on the oxidation reduction potential of NO₂ in neutral aqueous solutions, it has been postulated that NO₂ will exhibit a high degree of selectivity of reaction with the molecular constituents of living cells (Prutz et al. 1985). In a previous study, NO₂ generated by gamma radiolysis of aqueous NO₂⁺/NO₃⁻ solutions was found to oxidize tyrosine residues to biphenolic dimers accompanied by the formation of nitrotyrosine (Prutz et al. 1984). Subsequent studies by Prutz and colleagues applying pulse radiolysis techniques demonstrated selective tyrosine modification by NO₂ in three proteins and a peptide (Prutz et al. 1985).

Due to the large surface area of the lung and its role in gas exchange, this organ is the body’s greatest point of exposure to volatile air pollutants. Although NO₂ can react with other species in cigarette smoke (Church & Pryor, 1985) and potentially with biopolymers in the smoker’s lung (Pryor et al. 1982), little knowledge exists concerning the possible reaction of NO₂ with structural proteins such as
collagen and elastin (Morrow, 1984). Because emphysema is generally thought to result from an imbalance in the proteinase/proteinase inhibitor system (Thurlbeck, 1984), little attention has been paid to the possibility that agents such as NO₂ may alter the structure of elastin, resulting in direct damage or its increased susceptibility to proteolysis. Also, NO₂ may damage other proteins, such as α₁-PI, that protect the lung or play vital roles in its function. In support of the direct effects of oxidants, such as NO₂, on the integrity of elastin are reports that other oxidants and free-radicals can cause cleavage of peptide bonds in connective tissue proteins including fibronectin and collagen (Vartio, 1989; Curran et al. 1984). Additionally, ozone exposure of collagen was shown to increase its susceptibility to proteolytic degradation (Curran et al. 1984). To better understand the possible effects of NO₂ on the structure and function of α₁-PI and elastin in particular, and proteins in general, in vitro exposures have been conducted under highly controlled conditions at physiological pH. The results of these experiments led to similar experiments with poly-L-lysine and serum albumins. These data provide evidence for the reaction of NO₂ with lysine residues and the generation of peptides. The results of this research will contribute information that might be valuable in terms of understanding how proteins (in general) are altered by oxidant gases such
as NO₂, and perhaps provide insight as to the mechanism by which NO₂ induces lung injury.
Chapter 2
MATERIALS AND METHODS

[\textsuperscript{3}H]-Formaldehyde (5 \mu Ci/\mu l) was purchased from New England Nuclear; Bovine ligamentum nuchae elastin was obtained from Elastin Products Co.; Nitrogen dioxide (560 ppm) in Argon was purchased from Union Carbide Industrial Gases, Inc.; Bovine Serum Albumin (BSA; Biotech grade), sodium hydrogen phosphate (trihydrate), sodium salicylate, sodium acetate (enzyme grade), ammonium bicarbonate, acetonitrile (HPLC grade), acetic acid (ACS grade), hydrogen peroxide (30%), 2-(4-Morpholino)-Ethane Sulfonilic Acid (MES), and methanol (Optima) were obtained from Fisher Scientific Corp.; Amino acid standard H, triethylamine (Sequanal grade), and phenylisothiocyanate (PITC) were products of Pierce Chemical Co. Methanesulfonic acid (MSA), N-1-Naphthylethylenediamine (NEDA), Sulfanilamide (p-Aminobenzenesulfonamide), methoxysuccinyl-L-(Ala)\textsubscript{2}-Pro-Val-\textit{p}nitroanilide (pNA), N-benzoyl-L-Arg-pNA, succinyl-L-(Ala)\textsubscript{3}-pNA, porcine pancreatic elastase (PPE), porcine pancreatic trypsin (PPT), sodium cyanoborohydride, trichloroacetic acid (TCA), and poly-L-lysine (M\textsubscript{r} range = 30-70,000; for an assumed average M\textsubscript{r} of 50,000) were purchased from Sigma Chemical Corp. 4-chloro-1-naphthol was purchased from Polysciences, Inc. Phosphoric Acid was obtained from Baker Chemical Company. Dimethyl Sulfoxide (DMSO) 99%
spectrophotometric grade was purchased from Aldrich Chemical Company. DEAE Ion Exchange Spectra Gel was a product of Spectrum Medical Industries. Cellulose filtration membranes (0.45 micron pore size) were purchased from MSI. Nitrocellulose (0.2 μm pore size) was a product of Schleicher & Schuell Company. Pre-stained high M, marker protein standards used for SDS-PAGE were purchased from Diversified Biotech Company. PICO-TAG reaction vial and amino acid analysis column 3.9 mm x 15 cm with C-18 packing material that was end-capped were products of Waters International. A Pharmacia 4 x 125 mm HPLC cartridge column with 3 μm C-18 packing material and guard cartridge was used for PITC amino acid analysis and Sephadex chromatography media, G-25 and G-50, were from the same. Acrylamide and other chemicals for electrophoresis were obtained from BioRad. Cytoscint, liquid scintillation cocktail was a Beckman product. Human serum albumin was purified as previously described (Travis et al. 1976). Primary monoclonal antibodies—mouse anti-human alpha-1-proteinase inhibitor—and secondary antibodies—goat anti-mouse IgG horseradish peroxidase were gifts from Dr. S. K. Chan, University of Kentucky Medical Center. Human neutrophil elastase (HNE) was a gift from Dr. David A. Johnson. Trasylol was a gift from Bayer AG. The low M, "Rainbow" marker proteins from Amersham International were a gift from Dr. M. L. Ernst-Fonberg.
Purification of Alpha-1-Proteinase Inhibitor from Plasma

Lipid free outdated blood bank plasma donated from the American Red Cross, Johnson City, TN was used as the primary source of Alpha-1-Proteinase Inhibitor ($\alpha_1$-PI). The purification of $\alpha_1$-PI described is a modification of the procedure of Travis and Johnson (1981). Pooled plasma (typically 250 ml) was diluted with an equal volume of 0.05 M Tris-HCl, 0.05 M NaCl pH 8.5, 1 mM EDTA. The buffered plasma was brought to 40% ammonium sulfate saturation by the slow addition of solid ($\text{NH}_4$)$_2$SO$_4$ with constant stirring. The mixture was stirred a 4°C for 30 minutes, centrifuged at 12,000 x g for 30 minutes, and the precipitate was discarded. The supernatant fraction was then brought to 75% saturation by addition of solid ($\text{NH}_4$)$_2$SO$_4$, stirred at 4°C for 30 minutes, and re-centrifuged at 12,000 x g for 30 minutes. The precipitate obtained was redissolved in 20 ml of 0.05 M Tris-HCl pH 8.5 and dialyzed against 4 liters of the same for 24 hours at 4°C with 4 changes of the buffer.

The basic principle of the purification procedure involves the removal of albumin from ammonium sulfate-fractionated plasma by affinity chromatography on the dye-gel conjugate Cibacron Blue Sepharose (CBS). Cibacron Blue F3GA was coupled to Sepharose CL-4B as described by Travis and Johnson (1981). The dialyzed fraction (92 ml) was applied to a column of CBS (5 x 46 cm) equilibrated with 0.05 M Tris-HCl, pH 8.5, 1 mM EDTA at 25°C, washed with
equilibration buffer, and collected in 10 ml fractions at 130/ml hour. The fractions containing α₁-PI detected by inhibitory activity assays as described below were eluted in the void volume, while albumin remained tightly bound to the column. Collection of fractions was continued until the absorbance at 280 nm was below 0.05. The column was then stripped of albumin using 500 ml of 0.5 M KSCN. The stripped human serum albumin (HSA) was collected in 15 ml fractions, dialyzed extensively against H₂O, lyophilized, and stored at -15° C. The purity of this HSA was assessed by electrophoresis and was found to be 95% homogeneous. Two additional steps were required to completely purify α₁-PI.

Thiol-disulfide interchange chromatography using glutathione Sepharose (GSH-Sepharose), prepared by coupling GSH to cyanogen bromide activated Sepharose CL-4B, was used as the first step in the further purification of α₁-PI. The GSH-Sepharose gel was reacted with 200 mg of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to form the Sepharose G-S-S-5-thio-2-nitrobenzoic acid conjugate. The column was then washed with 0.05 M Tris-HCL, pH 8.0. Fractions from the CBS column containing α₁-PI were pooled, adjusted to pH 8.0, and incubated with the oxidized GSH-Sepharose gel for 15 hours with gentle shaking. Alpha-1-PI binds to this column intermolecularly via its reactive cysteine SH group with the glutathione SH group, giving a disulfide bond. The Sepharose-G-S-S-α₁-PI gel was then poured in a column (3 x
20 cm) and washed with 0.05 M Tris-HCl, pH 8.0, until the
A_{280} was less than 0.01, and eluted with 20 mM Cysteine in
the buffer, and 10 ml fractions were collected at a flow
rate of 250 ml/hour. These fractions were bright yellow due
to the elution of excess NBS from the Sepharose-G-S-S-NB
conjugate. Active fractions, determined by elastase
inhibitory activity assays, were pooled and dialyzed at 4\(^\circ\) C
against 4 liters of 20 mM MES pH 6.5 containing activated
charcoal to absorb the yellow NBS (half molecules of DTNB)
for 24 hours with 4 changes of the buffer. After dialysis,
the pooled fractions were applied to a column of DEAE-Ion
Exchange Spectra Gel (1 x 21 cm) equilibrated with 20 mM MES
pH 6.5, washed until the A_{280} was less than 0.01, and eluted
with a linear gradient of 0-0.2 M NaCl in 600 ml of
equilibration buffer, at a flow rate of 150 ml/hour.
Fractions containing \(\alpha_1\)-PI inhibitory activity were pooled,
dialyzed against 0.1 M NaH\(_2\)PO\(_4\) pH 7.4, and concentrated to an
A_{280} of 0.534 in an Amicon concentrator with a YM-30
filtration membrane and stored frozen in 1 ml aliquots at
-80\(^\circ\) C.

**Electrophoresis of \(\alpha_1\)-Proteinase Inhibitor**

The preparation was judged to be homogeneous by
discontinuous sodium dodecyl sulfate polyacrylamide gel
electrophoresis (SDS-PAGE) under reducing conditions, using
the amidiol buffer system of Bury (1981). Electrophoresis
was carried out on 1.5 mm 10\% resolving gels, using a BioRad
Model 360 Mini Vertical Slab Gel Apparatus and a Hoefer power supply. Electrophoresis started at 12 mA (45 volts) during stacking, changing to 20 mA (85 volts) after the tracking dye had entered the separating gel. The gels were stained with Coomassie Brilliant Blue containing formaldehyde, according to Steck et al. (1980), and destaining was performed using methanol, water, and acetic acid (5:5:1).

Inhibitory Activity Assays

Assays to detect inhibitory activity are based on the decrease in proteolytic activity of an enzyme resulting from preincubation with an inhibitor. Enzyme and inhibitor incubation times were based on half-times of association and the concentration of proteinase used in the reaction (Bieth, 1980). The inhibitory activities of α1-PI samples were monitored using PPE or HNE. Generally, the inhibitor was preincubated with the proteinase for 10 times the normal half-time of association \((t_{1/2} = 1/K_m [E^0])\) in a total volume 980 µl of buffer. Porcine pancreatic elastase and human neutrophil elastase activities were assayed using the synthetic substrates, Suc-(Ala)₃-pNA (Bieth et al. 1974), and/or Methoxy succ-(Ala)₂-Pro-Val-pNA (Castillo et al. 1979), respectively. All assays were conducted with an initial substrate concentration of 0.5 nmoles in a 1 ml assay. After preincubation, 20 µl of the appropriate substrate was added to the 980 µl preincubation and the
yellow color of the pNA derivative was allowed to develop for 15 minutes. The reaction was quenched by the addition of 50 µl of glacial acetic acid. The decrease in substrate hydrolysis, which is indicative of α₁-PI inhibitory activity relative to a control containing only elastase, was monitored by measuring the 410 nm absorbance of the pNA product in a Beckmann DU-30 Spectrophotometer. Final pooled, α₁-PI inhibitory activity was assayed in 0.1 M HEPES, 0.2 M NaCl pH 8.0, 0.05% Brij, 0.02% NaN₃ buffer using either porcine pancreatic trypsin (0.6 mg/ml in 2 mM HCl, 1mM CaCl₂) and the substrate Benzoyl-Arginine-pNA (Erlanger et al. 1961) or human neutrophil elastase (0.2 mg/ml) and the substrate Methoxysucc-(Ala)₂-Pro-Val-pNA (Castillo et al. 1979). All substrate stock solutions were prepared at a concentration of 20 mM in pure DMSO.

Labelling of Elastin

Insoluble bovine ligamentum nuchae elastin was labeled by reductive methylation according to the method of (Jentoft and Dearborn, 1979). Briefly, 100 mg of elastin was suspended in 5 ml of 0.1 M phosphate pH 7.4 that contained 6 mg/ml NaCNBH₃. To this suspension 12 µl of ³H-HCHO (5 µCi/µl) was added and the solution was incubated overnight at 37°C. ³H-labeled elastin was collected by centrifugation and washed with the buffer until less than 1% of the associated radioactivity could be detected in the supernatant fraction of the washes. The preparation used in
these studies had a radiospecific activity of \(1.81 \times 10^4\) CPM/mg and was stored at -4°C.

**Labelling of Bovine Serum Albumin (BSA), Human Serum Albumin (HSA), and Poly-L-Lysine**

The albumins (1 mg/ml, 15 \(\mu\)M) were dissolved in 200 \(\mu\)l of 0.1 M phosphate pH 7.4, containing 6 mg/ml NaCNBH₃. After adding \(^3\text{H}-\text{HCHO}\) (100 \(\mu\)Ci/50 mg BSA; 75 \(\mu\)Ci/20 mg HSA), these solutions were incubated for 4 hrs at 37°C on a shaking water bath. Albumin solutions were then chromatographed on a 2 x 12 cm Sephadex G-25 gel filtration column to remove unbound \(^3\text{H}-\text{HCHO}\). The labeled albumins eluted in the void volume, and unreacted \(^3\text{H}-\text{HCHO}\) eluted in the total volume of the column. Fractions containing either BSA or HSA were pooled, lyophilized, and redissolved at concentrations of 10 mg/ml in 0.1 M sodium phosphate buffer pH 7.4 and stored at -20°C until needed. Radiospecific activities of \(1.12 \times 10^3\) CPM/\(\mu\)g and \(3.8 \times 10^3\) CPM/\(\mu\)g were obtained for \(^3\text{H}-\text{BSA}\) and \(^3\text{H}-\text{HSA}\) preparations, respectively. Poly-L-lysine (5 mg) was similarly labeled with 100 \(\mu\)Ci of \(^3\text{H}-\text{HCHO}\), but no unbound \(^3\text{H}-\text{HCHO}\) was detected upon chromatography on Sephadex G-25. The labeled poly-L-lysine containing fractions were pooled, lyophilized, and resuspended at a concentration of 2.5 mg/ml (8.87 \(\times\) \(10^3\) CPM/\(\mu\)g) in the 0.1 M sodium phosphate pH 7.4 exposure buffer. This preparation was exposed to NO₂ at a concentration of 80 \(\mu\)g/ml.
In Vitro Exposure of Proteins and Peptide to NO₂

The exposure apparatus (Figure 3) used for exposure of proteins and peptides to NO₂ was constructed using Pyrex culture tubes (18 x 150 mm), stainless steel couplers, Neoprene rubber stoppers, Teflon tubing, and a gaseous flowmeter. Prior to exposure the amount of NO₂ entering the sample was quantified by reaction with an arsenite absorbing solution according to the method of Saltzman (1954) (EPA Method No. EQN-1277-026). In this procedure, NO₂ bubbled through a sodium arsenite-sodium hydroxide solution formed a stable solution of sodium nitrite. The nitrite ion produced during sampling was then treated with phosphoric acid, sulfanilamide, NEDA, and hydrogen peroxide. The resulting azo dye was measured colorimetrically at 540 nm and referenced to a standard curve of A₅₄₀ nm versus nanomoles of sodium nitrite.

NO₂ (250 ppm in N₂) was then bubbled at a flow rate of 5 nmoles/min through 5 ml solutions containing 53 μg/ml α₁-PI (1 μM, 5 nmoles) or 0.2 mg/ml α₁-PI (4 μM, 20 nmoles) buffered in 0.1 M NaH₂PO₄ pH 7.4 at a gaseous flow rate of 2.4 ml/minute; or NO₂ (560 ppm in Argon; 16 moles/ml of gas) was then bubbled through 5 ml solutions containing 1 mg/ml Elastin, BSA or HSA (15 μM, 75 nmoles) or 80 μg/ml poly-L-lysine (1.6 μM, with respect to N-termini based on an average M₅₀ of 50 kDa); a total of 3.2 μmoles (625 nmoles/ml of lysine side chain residues) buffered in 0.1 M NaH₂PO₄ pH
Carry-over

Downstream trap of absorbing solution (Traps unreacted NO₂)

Absorbing or Protein solution

MAGNETIC STIRRER

FLOWMETER

NO₂ Gas in Argon

Figure 3. In Vitro Exposure System.
7.4 at a gaseous flow rate of 2.4 ml/min. 1-Octanol (50 µl) was added to BSA, HSA, and poly-L-lysine samples prior to exposure to prevent bubbling, and all solutions were magnetically stirred. Following exposure the pHs of these solutions were unchanged.

A trap of absorbing solution was employed downstream from the sample to quantify unreacted NO₂, allowing quantification of the amount of NO₂ reacting with the target as well as the amount introduced into the protein/peptide solutions. Based on the conditions used, this resulted in a delivery of 38.4 nmoles of NO₂ per minute, but only 70% of the entering NO₂ reacted with the target peptides, based on the difference between the amount of NO₂ entering the peptide solution and the amount of NO₂ found in the downstream trap. Thus, the rate of NO₂ reactivity with the peptides was 27 nmoles/minute and the molar ratios of NO₂:protein given refers to moles of NO₂ reacted per mole of protein or mole of lysine residues. Control α₁-PI samples were bubbled with N₂ for the maximum exposure time (30 hours equals 800:1 molar ratio of NO₂:α₁-PI) to compensate for denaturation due to bubbling, these control samples lost less than 20% of the inhibitory activity of unbubbled α₁-PI, so the activity of the controls were taken as 100% in inhibitory activity assays. After exposure to NO₂ the α₁-PI samples were assayed for inhibitory activity then stored at 80⁰ C. The elastin, BSA, HSA, and poly-L-lysine control
samples were bubbled with argon carrier gas to standardize the loss of label due to physical treatment for the maximum exposure time (72 or 96 hours). Exposures were carried out under a chemical fume hood at ambient temperatures. Data are reported in terms of the moles of NO₂ reacting with the protein or peptide targets.

Immunoenzyme Assays

The quantitative immunoenzyme dot-blot assays were performed essentially according to the method of Smith et al. (1989). Generally, nitrocellulose (0.2μ pore size) was cut into 9 x 11 cm sheets and wetted in dH₂O at 75° C. The nitrocellulose filter was then incubated in 40 ml of 20 mM Tris-HCl, 0.15 M NaCl, (Tris buffered saline, TBS) pH 7.4 for 15 minutes to equilibrate the membrane with the TBS.

Control and NO₂ exposed α₁-PI was diluted to a concentration of 20 μg/ml in 0.025% SDS, and the samples were boiled for 5 minutes. Samples were then applied by gravity filtration in a BioRad Bio-Dot 96 well filtration apparatus until complete. Approximately 45 minutes was needed for a 80 μl sample. Each well was then washed by vacuum filtration with 200 μl of TBS. The unoccupied binding sites on the filter were blocked with a solution of BSA (3% w/v) in TBS for 3-5 hours with gentle shaking. The blocking solution was then replaced with a 1:10,000 dilution of primary monoclonal antibody or a 1:2,000 dilution of primary polyclonal antibody in 40 ml of TBS containing 1%
BSA, 0.025% SDS (antibody buffer) and incubated for 6 hours. The nitrocellulose filter was then washed 3 x 10 minutes in TBS. The secondary, peroxidase linked antibody (1:2,000 dilution for monoclonal and polyclonal) was incubated with the filter for 12 hours in the antibody buffer and washed as before. A fresh peroxidase substrate solution was then prepared by dissolving 60 mg of 4-chloro-1-naphthol in 20 ml of ice cold methanol which was added to 100 ml of 20 mM Tris-HCl, 0.5 M NaCl, pH 7.5, with stirring. Immediately before the substrate solution was added to the filter, 60 µl of 30% hydrogen peroxide was added. The color development was allowed to proceed until such a time that the dots were sufficient in intensity to photograph with a minimum background. The development reaction was stopped by flushing the filter with dH₂O. The filter was then photographed using Polaroid Type 55 positive/negative film and quantified by transmittance densitometric scanning of the photographic negative. A Hoefer densitometer was used in the quantification with an IBM computer and GS-360 Data system software (Metagraphics Software Corp., Scotts Valley, CA).

Amino Acid Analysis

Alpha-1-PI, soluble and insoluble ³H-Elastin, and ³H-poly-L-lysine samples were hydrolyzed in vacuo for 24 hours with 4 M Methanesulfonic acid (MSA) at 110°C, essentially according to the method of Simpson et al. (1976). The
quantitative analysis of the amino acids serine, threonine, tyrosine, and methionine is complicated by their instability to hydrolysis in HCl. The more common methods of HCl hydrolysis employ additives that protect against destruction such as thioglycolic acid. These common methods use a nonvolatile constituent that cannot be readily removed from protein/peptide hydrolyzates, which can result in possible interference in the derivatization reaction. The MSA method has the advantage in that it is a milder hydrolysis and the acids’ nonvolatility is easily dealt with by neutralization after hydrolysis. This translates into higher recoveries for these amino acids: Generally, 100 µl (400 pmoles) of Control and 800:1 NO₂ exposed α₁-PI, or the volume of ³H-Elastin (Soluble and Insoluble fractions) that gave approximately 1,000 counts per minute was dried in 6 x 50 mm sample vials by lyophilization. To the dried samples 20 µl of 4 M MSA was added, and the samples were hydrolyzed as described above. Following neutralization with 4 M KOH, samples were re-dried from 40 µl of MeOH:dH₂O:TEA (2:2:1 v/v), derivatized with 20 µl of MeOH:dH₂O:TEA:PITC (7:1:1:1 v/v), and dissolved in 200 µl of 40 mM sodium acetate, 0.05% TEA at pH 6.5. The α₁-PI hydrolyzates were redissolved at a concentration of 400 pmoles/200 µl diluent which equals 2 pmoles/µl and 25 µl (50 pmoles α₁-PI) of each hydrolyzate was injected for analysis. The ³H-Elastin (Soluble and Insoluble) hydrolyzates were redissolved in 200 µl diluent,
and 20 μl was injected for analysis. The HPLC column was standardized with 500 pmoles of PITC amino acids that were derivatized as described before. Chromatography and detection was carried out on Perkin-Elmer LC 135 with a Diode Array Detector at 254 nm or a Waters Model 510 Pump with a Series 440 Absorbance Detector at 254 nm. Data was processed using a Hewlett-Packard 3396A Integrator or a Waters 740 Data Module. The column temperature was maintained at 45° C. The following solvents were used for chromatograph: 40 mM sodium acetate, 0.05% TEA, pH 6.5 (Eluent A), and 70% acetonitrile, 30% Eluent A (Eluent B). A convex gradient from 87% A to 13% B for 7 minutes, then to 35% B for an additional 6.5 minutes was applied to elute the amino acids at a flow rate of 1 ml/minute. The elution position of ³H-dimethyllysine was determined by hydrolysis of the reductively methylated ³H-poly-L-lysine, followed by chromatography. After hydrolysis and PITC derivitization, both control and 72 hour NO₂ exposed ³H-poly-L-lysine samples yielded single peaks at the normal position for lysine with greater than 90% recovery of radioactivity in that peak.

**Electrophoresis and Fluorography of ³H-BSA and ³H-HSA**

SDS-PAGE analysis of ³H-BSA and ³H-HSA after exposure to NO₂ was carried out on 1.5 mm 10% polyacrylamide gels under reducing conditions, using the amidiol system of Bury, (1981). Proteins and peptides were stained with Coomassie Brilliant Blue containing formaldehyde and destained
according to Steck et al. (1980). Generally, the destained gels were soaked in 1 M sodium salicylate pH 6.2 for 30 minutes then dried under vacuum (BioRad Model 543 Gel Dryer) on Whatman 3MM paper under an acetate sheet for 2 hours. This is a modification of the method of (Chaimberlain et al. 1979). The positions of the standards were marked with a pen containing \(^{14}C\)-Toluene and the dried slab gel was placed against Kodak SB-5 film for 2 weeks at \(^{80}C\). The developed films were compared with the dried slab gels to demonstrate the generation of small peptides as a result of NO\(_2\) exposure.

**Gel Filtration Chromatography**

Chromatography of \(^3\)H-Poly-L-Lysine and \(^3\)H-BSA, before and after exposure to NO\(_2\) for 72 hours, was performed on 1.0 x 37 cm columns of Sephadex G-25 or Sephadex G-50, respectively. The columns were equilibrated with 0.2 M ammonium bicarbonate pH 8.2. Fractions of 300 \(\mu\)l were collected at a flow rate of 150 \(\mu\)l/min. Half of the volume was counted in liquid scintillation cocktail (Cytoscent) and the remaining volume was diluted 1:2 in dH\(_2\)O and the absorbance at 214 nm was monitored in the control and in the 72 hour samples. Greater than 95% and 97% of the samples were recovered for \(^3\)H-BSA and \(^3\)H-poly-L-lysine, respectively.
Purification of Alpha-1-Proteinase Inhibitor

The major steps in the purification of $\alpha_1$-PI from human plasma were ammonium sulfate precipitation, followed by chromatography on Cibacron Blue Sepharose (CBS), GSH-Sepharose, and DEAE Spectra Gel. A crucial step in the fractionation procedure is the removal of albumin which binds to the CBS column whereas $\alpha_1$-PI does not bind and elutes in the void volume. The exact nature of the binding of albumin to the reactive triazene dye Cibacron Blue is unknown. However, it is generally believed that electrostatic and hydrophobic interactions account for the interaction via the multiple aromatic and sulfonate groups on the dye molecule (Scopes, 1982). The $\alpha_1$-PI, which contains a reactive thiol group that reacts with NBS conjugated GSH-Sepharose displaces the NBS forming a disulfide bond with $\alpha_1$-PI. Other proteins with less reactive thiols were washed through the column, and elution of $\alpha_1$-PI was accomplished by reduction with cysteine. Ion-exchange chromatography on Spectra Gel DEAE was used to separate $\alpha_1$-PI from the remaining contaminants. The total yield of $\alpha_1$-PI in a typical preparation was 66\% with a concomitant 235-fold purification from ammonium sulfate fractionated plasma (Table II). From 250 ml of plasma, a
# TABLE II
## PURIFICATION OF HUMAN $\alpha_1$-PI

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein (mg)</th>
<th>Total Activity units</th>
<th>Specific Activity units/mg</th>
<th>Recovery (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>16.300</td>
<td>1,100</td>
<td>0.0675</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Dialyzed (NH$_4$)$_2$SO$_4$, 40-75% Sat. Precipitate</td>
<td>4,665</td>
<td>1,032</td>
<td>0.221</td>
<td>94</td>
<td>3.2</td>
</tr>
<tr>
<td>Cibicron Blue Sepharose</td>
<td>1,113</td>
<td>1,025</td>
<td>0.921</td>
<td>93</td>
<td>13.6</td>
</tr>
<tr>
<td>GSH-Sepharose</td>
<td>186.56</td>
<td>940.3</td>
<td>5.04</td>
<td>75</td>
<td>74.6</td>
</tr>
<tr>
<td>DEAE Ion Exchange</td>
<td>46.2</td>
<td>734.5</td>
<td>15.9</td>
<td>66</td>
<td>235.5</td>
</tr>
</tbody>
</table>
total of 46 mg of $\alpha_1$-PI was isolated based on a extinction coefficient of 5.3 for a 1% solution of $\alpha_1$-PI (Travis & Johnson, 1981).

Reduced SDS-PAGE of fractions eluted from the Spectra Gel DEAE Ion Exchange chromatography step showed one major band corresponding to a relative molecular weight of 53,000, in close agreement with the published value of 52,000 (Travis & Johnson, 1981).

**Molar Stoichiometry**

The amount of active $\alpha_1$-PI in the total DEAE pool was determined by calculation of the molar stoichiometry of the inhibitor with proteinases. This was accomplished by incubating increasing amounts of the inhibitor with either PPE or HNE. The residual enzyme activity was determined and plotted versus the molar ratio of enzyme to inhibitor added (Figure 4). At zero enzyme activity (i.e., 100% inhibition) the enzyme to inhibitor ratio necessary for 100% inhibition was calculated. Alpha-1-PI was shown to inhibit PPE at an enzyme:inhibitor ratio of 1.0:0.98, which indicated that the inhibitor was 98% active against PPE. Inhibition of HNE was shown to occur at a ratio of 1.0:1.0, indicating that the inhibitor is 100% active against HNE.
Figure 4. Titration of Proteases with α₁-PI. Increasing amounts of α₁-PI were incubated with (A) (7.4 μM) HNE or (B) (8.8 μM) PPE followed by assay of residual enzymatic activity.
Effects of NO₂ on the Activity and Immunoreactivity of α₁-PI.

Alpha-1-PI was exposed to NO₂ by bubbling at a flow rate of 9.7 nmoles NO₂ per minute at a gaseous flow rate of 2.4 ml/min. Following NO₂ exposure, the inhibitory activity of α₁-PI was assayed with HNE. A plot of percent activity versus moles of NO₂ reacted per mole α₁-PI is shown in Figure 5. The data points represent the average of 4 assays. The inhibitory activity of α₁-PI was found to decrease as the molar ratio of NO₂:α₁-PI increased. At a molar ratio of NO₂:α₁-PI of 800:1, the inhibitory activity of α₁-PI decreased to 50% of the control value.

The stability of complexes formed between NO₂ treated α₁-PI and HNE were analyzed electrophoretically as shown in Figure 6. Native α₁-PI (lane 2) with a molecular weight of 53,000 formed a stable complex with HNE (lane 1), molecular weight 30,000 Barrett (1981), which gives a total α₁-PI:HNE complex molecular weight of 78,000 (lane 3), due to the release of a 5,000 Mᵣ fragment from the C-terminal portion of α₁-PI upon reaction with proteinases (Travis & Salvesen, 1983). With increasing molar ratios of NO₂:α₁-PI, the inhibitor seems to lose a portion of its ability to form a stable complex with HNE. This can be seen as a slight increase in the residual HNE as the molar ratio of NO₂:α₁-PI increases. Concurrent with this increase in residual HNE,
Figure 5. The Effect of NO$_2$ on the Inhibition of HNE by $\alpha_1$-PI. NO$_2$ was bubbled through 20 nmoles (1040 µg) of $\alpha_1$-PI in 5 ml of 0.1 M NaH$_2$PO$_4$ pH 7.4 at a flow rate of 9.7 nmoles NO$_2$/min for up to 30 hours. Corresponding to molar ratios of NO$_2$:$\alpha_1$-PI of 100:1, 200:1, 400:1, and 800:1. NO$_2$ exposed $\alpha_1$-PI was assayed by measuring its inhibition of HNE activity following preincubation of enzyme and inhibitor for 1 hour. Each data point represents the average of 4 assays and error bars indicate the standard error of the mean.
Figure 6. Electrophoresis of α₁-PI:HNE Complexes. Native and NO₂ exposed α₁-PI were incubated with HNE at a 1:1 molar ratio of enzyme to inhibitor for 30 minutes at pH 7.4. Incubations were stopped by TCA precipitation and 20 µg of protein was loaded in each well after treatment with SDS sample buffer. Lane 1 Native HNE, Lane 2 Native α₁-PI, Lane 3 Native α₁-PI + HNE, Lane 4 100:1 + HNE, Lane 5 200:1 + HNE, Lane 6 400:1 + HNE, Lane 7 800:1 + HNE.
there appears to be a decrease in the amount of complex formation as the NO₂ concentration increases.

To examine whether NO₂ exposure of α₁-PI caused a loss of its immunological reactivity, immuno dot-blot assays were conducted with polyclonal and monoclonal antibodies. Figure 7 is a representative dot-blot, which was quantified by densitometric scanning of the photographic negative and the data are graphically depicted in Figure 8. The results in Figures 7 and 8 indicated that with increasing molar ratios of NO₂:α₁-PI the immunoreactivity of α₁-PI decreases, relative to control α₁-PI. Essentially, identical results were obtained with either monoclonal or polyclonal antibodies; with a 50% loss in the immunoreactive response at molar ratios of 100:1 and greater. The results of the inhibitory activity assays, the molar stoichiometry complex formation gels, and the immunoreactivity assays suggest that the loss of α₁-PI inhibitory activity and immunoreactive response may be related to a loss of soluble protein that occurs as a result of NO₂ exposure. To test this hypothesis in the dot-blot assay system, samples were filtered through 0.45 μ cellulose acetate membranes prior to application to the membrane, and a statistical analysis was performed on the linearity of the immunoreactive response at each exposure level over a range of concentrations. Since the monoclonal and polyclonal antibodies had given equivalent responses in previous assays and more of the polyclonal antisera was
Figure 7. Immunoenzyme Assay of Control and NO₂ Exposed α₁-PI (1 nmol/ml). Samples were made .02% in SDS and boiled for 5 minutes. Samples were then blotted onto nitrocellulose at the indicated concentrations and incubated with either mouse monoclonal or rabbit polyclonal antibodies followed by incubation with horseradish peroxidase conjugated secondary antibodies to mouse or rabbit IgG. The color development of the membrane was accomplished using 4-chloro-1-naphthol substrate.
Figure 8. NO$_2$ Exposed $\alpha_1$-PI Immunoreactivity. The percent response represents the immunoreactivity for control and NO$_2$ exposed $\alpha_1$-PI with polyclonal (---●---) and monoclonal (---□---) antibodies. This was calculated by dividing the peak areas for each dot in the 10 ng lane of Figure 9 by the control value.
available, the assay was performed with the polyclonal antibody. The resulting dot-blot is shown in Figure 9. The photographic negative of the blot in Figure 9 was quantitated by densitometric scanning. Statistical analyses were performed on the data after converting the responses for each concentration of $\alpha_t$-PI to a percent of the control at that $\alpha_t$-PI concentration (Figure 10). These data (Figures 9 and 10), using filtered $\alpha_t$-PI, samples show that approximately 40% of the immunoreactivity was lost with increasing exposure to NO$_2$. O-Phthalaldehyde (OPA) protein assays were performed, essentially according to the method of Peterson (1983) (Figure 11), on the filtered $\alpha_t$-PI samples to address the possibility that the loss of immunoreactivity was due to precipitation of $\alpha_t$-PI upon exposure to NO$_2$. A plot of the relative fluorescence resulting from the reaction of $\alpha_t$-PI samples with OPA relative to control $\alpha_t$-PI samples is similar to the inhibitory activity assays (Figure 5) as well as the immunoreactivity assays, (Figures 8 and 10) indicating that the decreases in $\alpha_t$-PI inhibitory activity and immunoreactivity most probably resulted from a loss of soluble protein upon exposure to NO$_2$.

With increasing exposure of $\alpha_t$-PI to NO$_2$, it was noted that the color of the solution changed from clear to slightly yellow, and a precipitate was barely visible after the highest exposure levels (30 hours). Centrifugation of
Figure 9. Immunoenzyme Assay of Control and NO$_2$ Exposed $\alpha_1$-PI (4nmol/ml). Samples were filtered then made .02% in SDS and boiled for 5 minutes and blotted onto nitrocellulose at the indicated concentrations. Rabbit polyclonal antibodies were incubated with the membrane followed by horseraddish peroxidase conjugated against Rabbit IgG secondary antibodies, followed by color development with 4-chloro-1-naphthol substrate.
Figure 10. Statistical Analysis of Control and NO2 Exposed α1-PI Immunoreactivity. Immunoreactivity was determined by dividing the peak heights obtained from scanning dot-blot by the concentration of α1-PI in the original sample before filtration. Percent immunoreactivity was calculated relative to the unexposed α1-PI control. Error bars represent the standard error of the mean for each exposure level.
Figure 11. Total Protein Determination on Control and NO₂ Exposed α₁-PI by the OPA Method. The fluorescence of each sample was measured relative to the control which was set to 100% relative fluorescence, with 340 nm excitation and 340 nm emission.
the 400:1 and 800:1 NO\textsubscript{2} exposed \(\alpha_{i}\)-PI samples at 5000 x g for 5 minutes revealed the presence of a yellow, insoluble material. This yellow precipitate was examined via SDS-PAGE and amino acid analysis. Figure 12 shows the electrophoretic pattern of the yellow, insoluble 400:1 and 800:1 precipitates after partial solubilization in 2% SDS. Note the presence of low molecular weight peptides in lanes 2, 3, 4, and 5, indicating the generation of small peptide fragments. There were no differences in the patterns from the precipitates resulting from exposure in the light (lane 4) vs. the dark (lane 5).

To obtain more of this precipitate \(\alpha_{i}\)-PI was exposed to NO\textsubscript{2} at a molar ratio of NO\textsubscript{2}:\(\alpha_{i}\)-PI of 1,600:1 (60 hrs). Amino acid analysis of native \(\alpha_{i}\)-PI and of the yellow, insoluble material from the 1,600:1 exposure are shown in Table III. The results revealed that the yellow precipitated material was \(\alpha_{i}\)-PI in which the values for the hydroxyl amino acids serine, threonine, and tyrosine were extremely low relative to native \(\alpha_{i}\)-PI. Also fewer lysine residues were found, as compared to the content based on sequence data (Carrell et al. 1982). Arginine appeared to have increased to 9 times the expected level. It is suspected that this increase in arginine represents a previously undescribed modified amino acid derivative that is eluting in the same position as arginine.
Figure 12. SDS-PAGE Analysis of the Yellow Insoluble Material Formed from NO₂ Exposure of α₁-PI. Lane 1 25 µg of Control α₁-PI, Lane 2 400:1 α₁-PI precipitate, Lane 3 800:1 α₁-PI precipitate, Lane 4 25 µg of 800:1 α₁-PI (Light reaction), Lane 5 25 µg of 800:1 α₁-PI (Dark reaction). Approximately 25 µg of protein was loaded in each well, except in Lanes 2 and 3 where the total precipitate was dissolved in 50 µl SDS sample buffer and 25 µl was loaded into wells. Molecular weight standards were applied to outermost lanes and were, as indicated, phosphorylase b (100 kDa), transferrin (78 kDa), BSA (67 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (21 kDa), cytochrome c (12.7 kDa), and Trasylol (6.4 kDa).
Table III. Amino Acid Analysis of MSA Hydrolyzates of Native $\alpha_1$-PI and of the Yellow, Insoluble, Precipitated Material Formed in the 1600:1 NO$_2$ Exposed $\alpha_1$-PI Sample.

<table>
<thead>
<tr>
<th>AA</th>
<th>Residues/mole Native</th>
<th>Residues/mole NO$_2$ Exposed</th>
<th>Residues/mole Res/mol$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>49</td>
<td>48.1</td>
<td>49</td>
</tr>
<tr>
<td>Glu</td>
<td>50</td>
<td>50.5</td>
<td>54</td>
</tr>
<tr>
<td>Ser</td>
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</tr>
<tr>
<td>Ile</td>
<td>18</td>
<td>22.1</td>
<td>19</td>
</tr>
<tr>
<td>Leu</td>
<td>50</td>
<td>47.5</td>
<td>50</td>
</tr>
<tr>
<td>Phe</td>
<td>27</td>
<td>27.3</td>
<td>28</td>
</tr>
<tr>
<td>Lys</td>
<td>35</td>
<td>27.3</td>
<td>39</td>
</tr>
</tbody>
</table>

* Based on the amino acid sequence (Carell et al. 1982).
Absorption spectra for native and NO$_2$ exposed α$_1$-PI are shown in Figure 13. Figure 13 (A) reveals that there is generally a 72% increase in the 280 nm absorbance of the NO$_2$ exposed α$_1$-PI samples relative to native α$_1$-PI. This absorbance increase was even greater after 72 hours of exposure with nitrogen carrier gas only, indicating that it was not due to NO$_2$ Figure 13 (scan f). The spectra reveal that there is a 4.7 fold increase in the 280 nm absorbance relative to native α$_1$-PI. To determine whether this was a general effect with proteins as opposed to a specific effect with α$_1$-PI; 1 mg/ml solutions of HSA (native), Figure 13 B(scan g): were bubbled with argon for 36 hours, Figure 13 B(scan h), and with N$_2$ for 36 hours, Figure 13 B(scan i). Clearly, the N$_2$ bubbled HSA showed a 37% increase in the 280 nm absorbance relative to native HSA, whereas argon bubbled HSA remained unaffected.

The identity of the peak at 356 nm present in the UV-Visible absorption spectra of the 400:1 and 800:1 NO$_2$ exposed α$_1$-PI samples (Figure 13 A) was determined to be sodium nitrite (NaNO$_2$) that is generated in situ by the reaction of NO$_2$ with NaH$_2$PO$_4$. Initially, the 356 nm absorption peak was believed to be representative of the acidic form of nitrotyrosine $\lambda_{\text{max}}$ 356 nm at pH 3.6 as shown by Prutz et al. (1985). However, amino acid analysis of these samples revealed no reduction in the tyrosine:glycine
Figure 13 (A). UV-Visible Absorption Spectra for Native and NO₂ Exposed α₁-PI (4 nmol/ml), and 72 hour. Nitrogen Bubbled Control α₁-PI (f) (4 nmol/ml). Absorption spectra were monitored at a scan rate of 1.0 nm/sec between 215 and 500 nm. (a) native α₁-PI, (b) 100:1, (c) 800:1, (d) 400:1, (e) 200:1.
Figure 13 (B). UV-Visible Absorption Spectra of 1mg/ml solutions of HSA. Absorption spectra were monitored at a scan rate of 1.0 nm/sec between 215 and 500 nm. (g) Native HSA; (h) Argon bubbled HSA; (i) Nitrogen bubbled HSA.
ratios (Table IV), which is an indicator of nitrotyrosine formation.

The *in vitro* effects of NO₂ on the activity and immunoreactivity of human α₁-PI that was purified to homogeneity in an active form from outdated blood bank plasma have been examined. The initial findings of a 50% reduction in inhibitory activity and immunoreactivity upon NO₂ exposure are suspected to result primarily from a loss of soluble protein due to precipitation in the *in vitro* exposure system. However, more interesting is the possibility that NO₂ has a direct oxidative effect, in general, on the structure of proteins. This possibility was examined further by studying the effects of NO₂ (in argon) on bovine ligamentum nuchae elastin, poly-L-lysine, and the serum albumins.

**TABLE IV.**

**AMINO ACID ANALYSIS OF CONTROL AND NO₂ EXPOSED α₁-PI**

<table>
<thead>
<tr>
<th>Ratio NO₂:α₁-PI</th>
<th>Ratio (residue/residue)</th>
<th>Tyr (Res/mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tyr:Gly</td>
<td>Tyr:Ala</td>
</tr>
<tr>
<td>CONTROL</td>
<td>0.257</td>
<td>0.188</td>
</tr>
<tr>
<td>800:1</td>
<td>0.285</td>
<td>0.215</td>
</tr>
</tbody>
</table>
Exposure of $^3$H-Elastin. The exposure of $^3$H-elastin to NO$_2$ for 9, 18, 36, and 72 hours resulted in the reaction of 15, 30, 60, and 120 μmoles of NO$_2$ with the target protein samples at the corresponding time points. Control samples were treated with argon for the same lengths of time. Elastin was collected by centrifugation at 10,000 x g for 15 minutes, giving soluble and insoluble fractions for each exposure level. The results of the amino acid analyses of both fractions are shown in Table V. With increasing exposure to NO$_2$, the total micrograms of $^3$H-dimethyllysine increased in the soluble fraction and a corresponding decrease was observed in the total micrograms of $^3$H-dimethyllysine present in the insoluble fraction. From amino acid analyses the percentages of $^3$H-dimethyllysine appearing in the soluble fractions at the various time points are displayed in Figure 14. These data demonstrate that 80% of the $^3$H-dimethyllysine residues in the insoluble control $^3$H-elastin were liberated into the supernatant after 72 hours of exposure or a total of 120 μmoles of NO$_2$. Other calculations, based solely on soluble radioactivity, led to a similar plot in Figure 14. These results, suggesting a preferential reactivity of NO$_2$ at lysine residues, led to exposure studies with $^3$H-poly-L-lysine as the target molecule.
Table V: $^3$H-Dimethyllysine Release From Control and NO$_2$ Exposed $^3$H-Elastin

<table>
<thead>
<tr>
<th>NO$_2$ Exposure (hrs)</th>
<th>$^3$H-Dimethyllysine$^b$</th>
<th>NO$_2$</th>
<th>Counts per minute*</th>
<th>(\mu)g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(\mu)moles</td>
<td>Soluble</td>
<td>Insoluble</td>
</tr>
<tr>
<td>CONTROL</td>
<td></td>
<td>0</td>
<td>4,968</td>
<td>87,766</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>15</td>
<td>28,161</td>
<td>62,212</td>
</tr>
<tr>
<td>18</td>
<td></td>
<td>30</td>
<td>30,420</td>
<td>43,232</td>
</tr>
<tr>
<td>36</td>
<td></td>
<td>60</td>
<td>45,486</td>
<td>34,776</td>
</tr>
<tr>
<td>72</td>
<td></td>
<td>120</td>
<td>73,773</td>
<td>18,186</td>
</tr>
</tbody>
</table>

*$^*$ Data are relative to the 5 mg $^3$H-Elastin sample that was exposed. $^b$ Determined by amino acid analysis.
Figure 14. $^3$H-Dimethyllysine Release from NO$_2$ Exposed $^3$H-Elastin. Based on the amino acid analysis of the soluble fraction after NO$_2$ exposure of $^3$H-Elastin, the total micrograms of $^3$H-dimethyllysine solubilized are displayed as a percentage of the insoluble control (---○---). Total solubilized radioactivity in counts per minute are also displayed as a percentage of the $^3$H-Elastin target sample for NO$_2$-exposed elastin (---■---) and similarly for the Argon bubbled control $^3$H-Elastin (---□---).
Exposure of $^3$H-poly-L-lysine. To investigate whether NO$_2$ was reacting at lysine residues, as suggested by the release of $^3$H-dimethyllysine from elastin, $^3$H-labeled poly-L-lysine (8 nmols of N-termini) was treated with NO$_2$ for 72 hours (120 μmols NO$_2$; 38 mols NO$_2$ per mole of lysine side chains). The use of this soluble target molecule allowed gel filtration chromatographic analyses of the polypeptide before and after exposure. Exposure of $^3$H-poly-L-lysine at a molar ratio of 15,000:1 (mols NO$_2$ : mols of peptide; 38:1 mols NO$_2$ : mole of lysine residues) resulted in extensive peptide bond cleavage of the native 50,000 M, peptide.

Figure 15 (A) shows the elution pattern of the argon-treated control $^3$H-poly-L-lysine and Figure 15 (B) shows the elution pattern of the products from the 72 hour NO$_2$ exposed sample. Fractions were monitored by liquid scintillation counting and absorbance at 214 nm to detect the radioactive label and peptide bonds, respectively. Native $^3$H-poly-L-lysine ($M_r=30-70,000$) eluted from the column within the void volume with a 95% recovery of the label, whereas the products from the 72 hour NO$_2$ exposed sample eluted within the internal column volume ($V_t$), with a 97% recovery of the label. Since NO$_2$ absorbs at 210 nm, the relatively large 214 nm absorbance peak in the $V_t$ region is probably due to both peptides and dissolved NO$_2$. Based on the fractionation range of Sephadex G-25, the peptides generated from the NO$_2$ exposure of $^3$H-poly-L-lysine are estimated to be in the
Figure 15. Gel Filtration Analysis of Control and NO₂ Exposed ³H-poly-L-lysine. (A) Elution profile of Control ³H-poly-L-Lysine (470 µg; 4.2 x 10⁶ CPM) on a Sephadex G-25 column (1 x 37 cm), previously equilibrated with 0.2 M ammonium bicarbonate buffer pH 8.1. (B) Elution profile from the same column of products produced by exposure of ³H-poly-L-lysine (11.2 µg; 1.0 x 10⁵ CPM) to NO₂ for 72 hours (120 µmoles). Fractions of 300 µl were collected at a flow rate of 150 µl/min, and one half of the volume was used for liquid scintillation counting (---●---). The remaining half was diluted 1:2 in dH₂O and the absorbance of the peptide bond at 214 nm was monitored (--■--).
Figure 15 (B).
M₄ range of 1-3,000. These results clearly demonstrate that exposure of \(^{3}\text{H}\)-poly-L-lysine to NO₂ caused substantial fragmentation of poly-L-lysine.

**Exposure of BSA.** Based on the previous results, indicating peptide bond cleavage at Lys residues in both elastin and poly-L-lysine, similar experiments were performed using serum albumins to determine whether the observed reactions occurred at all Lys residues or whether this reaction was limited in nature. Initially, 75 nmoles of \(^{3}\text{H}\)-BSA were exposed for 72 hours to 120 \(\mu\)moles NO₂ (1600:1; NO₂:BSA molar ratio), and samples were filtered through 0.45 micron cellulose acetate membranes prior to chromatography on Sephadex G-50. Figure 16 (A) shows the elution pattern of argon exposed control \(^{3}\text{H}\)-BSA and Figure 16 (B) shows the elution pattern of the NO₂ exposed sample. The native \(^{3}\text{H}\)-BSA eluted from the column within the void volume \((V₀)\), whereas after 72 hours of exposure to NO₂ products eluted at \(V₀\) and \(V₁\) indicating partial peptide bond cleavage of the native 66 kDa protein. Based on the CPMs recovered in each peak, 30% of the \(^{3}\text{H}\)-BSA eluted at \(V₁\) after NO₂ exposure.

An SDS-PAGE time course analysis of NO₂ effects on \(^{3}\text{H}\)-BSA is presented in Figure 17 (A). Five ml of a 1 mg/ml \(^{3}\text{H}\)-BSA solution in 0.1 M sodium phosphate buffer at pH 7.4 was exposed with NO₂ and samples were withdrawn at 12, 24, 36, 48, and 72 hours, resulting in corresponding NO₂:BSA molar
Figure 16. Gel Filtration Analysis of Control and NO₂ Exposed ³H-BSA (A) Elution profile of Control ³H-BSA (122 µg; 1.37 x 10⁵ CPM) was on a Sephadex G-50 column (1 x 37 cm), previously equilibrated with 0.2 M ammonium bicarbonate buffer pH 8.1. (B) Elution profile from the same column of the products produced by the exposure of ³H-BSA (90 µg; 1.0 x 10⁵ CPM) to NO₂ for 72 hours (120 µmoles). Fractions of 300 µl were collected at a flow rate of 150 µl/min. and one half of the volume was counted (—●—). The remaining 150 µl of sample was diluted 1:2 in dH₂O and the absorbance of the peptide bond at 214 nm was monitored (—■—).
Figure 16 (B).
Figure 17. SDS-PAGE Analysis of $^3$H-BSA After Exposure to NO$_2$. Lane 1, Control $^3$H-BSA (5 mg, 1.18 x 10$^3$ CPM/μg BSA) was treated with argon for the maximum exposure time. All remaining samples were treated with NO$_2$ at the indicated molar ratios of NO$_2$:$^3$H-BSA. Lane 2, 266:1, Lane 3, 533:1, Lane 4, 800:1, Lane 5, 1066:1, Lane 6, 1600:1. Approximately 40 μg of protein was loaded into each well. Molecular weight standards were applied to outermost lanes and were, as indicated, phosphorylase b (95 kDa), glutamate dehydrogenase (55 kDa), ovalbumin (43 kDa), lactate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), lactoglobulin (18.4 kDa), and cytochrome c (12.4 kDa). (A) Coomassie Blue Staining (B) Fluorography, developed after a two week exposure at -80°C.
rations of 266:1, 533:1, 800:1, 1066:1, and 1600:1. That the amount of BSA aggregation increased with time is evident from the Coomassie Blue staining (Figure 17 A) and from fluorography (Figure 17 B) of the electrophoresis gel. Increasing amounts of a yellow precipitate appeared as the exposures progressed and this material was suspended by vortexing prior to the removal of samples for SDS-PAGE, but the insoluble material was not removed. Additionally, labeled low $M_r$ fragments are visible in the fluorograph (Figure 17 B). Due to minor contaminants in the BSA the results are difficult to interpret, but peptides of less than 12.4 kDa appeared in all lanes upon exposure and the bands at 29 kDa seemed to be increasing in intensity with time.

To determine the extent of tyrosine nitration the yellow precipitate from the 72 hour time point NO$_2$ exposure of BSA (1600:1; NO$_2$:BSA molar ratio) was collected by centrifuging 1 ml (1 mg original $^3$H-BSA) of the suspension at 13,000 x g for 10 minutes. This material was dissolved by boiling for 10 minutes in 0.1 M sodium phosphate buffer at pH 7.4 containing 1% SDS and 2% beta-mercaptoethanol. A small amount of precipitate that would not dissolve was removed by filtration through a 0.45 micron cellulose acetate filter. The visible spectrum of this sample, after adjustment to pH 10.5 with 0.1 M NaOH, contained an absorption peak at 428 nm, corresponding to the published
Figure 17 (B).
maxima of nitrotyrosine (Prutz et al. 1985). Relative to a nitrotyrosine standard, 23% of the tyrosines in the 1 mg \(^{3}\text{H}\)-BSA sample had been converted to nitrotyrosine.

**Exposure of HSA.** To overcome the problem due to impurities in the BSA and to gain information on the nature of the observed precipitates, similar experiments were performed using highly purified \(^{3}\text{H}\)-HSA, with a maximum exposure time of 96 hours. Five ml of a 1 mg/ml \(^{3}\text{H}\)-HSA solution in 0.1 M sodium phosphate buffer at pH 7.4 was exposed with NO\(_2\) and samples were withdrawn for analysis at 24, 48, 72, and 96 hours, resulting in corresponding NO\(_2\):HSA molar ratios of 533:1, 1,066:1, 1,600:1, and 2,133:1. As with BSA exposures, a yellow precipitate appeared with time that was suspended in the buffer when samples were withdrawn. SDS-PAGE analysis of samples removed at the indicated times is shown in Figure 18. Precipitated material from the longest time was removed by centrifugation at 6,500 x g for 10 minutes and partially solubilized in SDS-PAGE sample buffer containing 2% SDS prior to electrophoresis. Approximately 15% of the supernatant fraction from the longest time was loaded in lane 7. Fluorography of this gel revealed aggregates and low M\(_{r}\) peptides at all times, but intermediate fragments were visible only with the precipitate (lane 6). The supernatant fraction (lane 7) appeared to contain primarily native HSA.
Figure 18. Fluorography Following 10% SDS-PAGE Analysis of $^3$H-HSA After Exposure to NO$_2$. Lane 1, Control $^3$H-HSA (5 mg, 3.8 x $10^3$ CPM/µg HSA) was treated with argon for the maximum exposure time. All remaining samples were treated with NO$_2$ at the indicated molar ratios of NO$_2$: $^3$H-HSA. Lane 2, 533:1; Lane 3, 1066:1; Lane 4, 1600:1; Lane 5, 2133:1; Lane 6, overload of precipitated $^3$H-HSA obtained by centrifugation of 200 µl of the 2133:1 exposure; Lane 7, 15% of the supernatant fraction obtained in the preparation of the sample in Lane 6. Approximately 25 µg of protein was loaded into Lanes 1 through 5, and the film was exposed at -80°C for 14 days. Molecular weight markers are the same as shown in Figure 17.
The precipitates resulting from the exposure of \(^3\)H-HSA were collected by centrifugation and washed twice with 0.1 M sodium phosphate buffer at pH 7.4 and twice with water to remove soluble components. Since treatment with the normal SDS-PAGE sample buffer had not resulted in complete solubilization of the precipitates, the concentration of SDS was increased to 5% and 1% DTT was used as the reducing agent. After boiling for five minutes the precipitates were dissolved. The Coomassie stained SDS-PAGE Analysis gel of these solubilized precipitates is shown in Figure 19 (A) and the fluorographic images resulting from SDS-PAGE of these solubilized precipitates are shown in Figure 19 (B), which can be seen to contain increasing amounts of aggregated and seemingly native HSA. Additionally, increasing amounts of peptides of approximately 46 and 6.5 kDa are evident in lanes 4 and 5.
Figure 19. Time Course SDS-PAGE Analysis of the Yellow, Insoluble Material Formed From NO₂ Exposure of ³H-HSA. Lane 1, Control ³H-HSA (25 μg; 3.8 x 10⁴ CPM/μg) after treatment with argon for the maximum exposure time. All remaining samples were treated with NO₂ at the indicated molar ratios of NO₂:³H-HSA. All samples were then centrifuged at 6,500 x g for 5 minutes to collect the insoluble material and the precipitates were washed twice with 0.1 M phosphate buffer pH 7.4, followed by two washes with H₂O. Argon exposed ³H-HSA did not contain a precipitate. The precipitates were then resuspended in 50 μl of the normal SDS sample buffer, which was 1% in both β-ME and SDS. When the precipitates did not dissolve upon boiling for 5 minutes, the precipitates were then made 5% and 2% in SDS and DTT, respectively, and boiled for an additional 5 minutes, which was sufficient to solubilize the yellow material. One half of the volume of each sample was analyzed. Lane 2, 533:1 precipitate; Lane 3, 1066:1 precipitate; Lane 4, 1600:1 precipitate; Lane 5, 2133:1 precipitate. Molecular weight markers were applied to outermost lanes and are, as indicated, ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.3 kDa), and Trasylol (6.5 kDa). (A) Coomassie Blue Staining (B) Fluorography, developed after a two week exposure at -80°C.
Figure 19 (B).
Chapter 4

DISCUSSION

General Aspects of Direct Oxidative Damage to Lung Proteins.

An in vitro system was used for the exposure of proteins to NO₂ in aqueous solutions at physiological pH. This approach was taken to mimic physiological conditions and to provide previously unavailable information on the reaction of NO₂ with proteins. The use of a gaseous NO₂ exposure system was chosen in preference to the generation of NO₂⁻ radicals by pulsed radiolysis as described by Prutz et al. (1985) because data are available on environmental concentrations of NO₂, which may allow some comparison of these results with environmentally relevant exposures, whereas concentrations of NO₂⁻ radicals are not measurable in terms of ambient air concentrations. Alpha-1-proteinase inhibitor and elastin were used as initial targets because of their critical role in lung function as evidence has shown that inhalation of NO₂ results in an emphysema-like insult to the lung. Direct oxidative damage to α₁-PI by ozone has been demonstrated previously (Johnson, 1980), and cigarette smoke has also been shown to inactivate α₁-PI (Pryor et al. 1984a). The gas phase of cigarette smoke has been shown to contain appreciable amounts of nitroxide species (Pryor et al. 1984b), so that the evidence for the role of oxidation in inactivating α₁-PI with cigarette
smoking seems to be incontrovertible (Carp et al. 1982). There seems to be only one previous report that addresses the direct effects of NO₂ on α₁-PI in an in vitro exposure system (Dooley & Pryor, 1982). In that study, 75 ppm NO₂ was bubbled through 0.25 mg/ml phosphate buffered protein solutions at a flow rate of 20 ml NO₂/minute for 2 hours. Upon assaying for α₁-PI inhibitory activity following exposure, no inactivation of α₁-PI was observed. However, when 1 mM H₂O₂ was added to the α₁-PI exposure reaction mixture, a total loss of inhibitory activity was observed. Conversely, studies in humans that were exposed to 3–4 ppm NO₂ for 3 hours revealed a 45% inactivation of the α₁-PI elastase inhibitory activity of their bronchoalveolar lavage fluids relative to air exposed control subjects (Mohsenin & Gee, 1987), but these results have been challenged (Johnson et al. 1990). The foregoing studies served as the impetus for the present study as they indicated that α₁-PI is a susceptible target for oxidation by ozone and NO₂ in the lung and that the inhalation of these pollutants could possibly lead to inactivation of this major proteinase inhibitor.

Modification of Tertiary Structure Following Exposure to NO₂.

Previous studies by Davies and Delsignore (1987b) have shown that a significant loss of solubility in KCl occurs when BSA is exposed to hydroxyl radical (OH·). Their in
vitro exposure system produced the hydroxyl radical in dilute protein solutions (0.33 mg/ml) by irradiation of water using 60Co, under 100% N2O. This decreased solubility was pH-dependent and was more pronounced at a radical:BSA molar ratio of 100:1 than at a ratio of 10:1. The decreased salt solubility close to the isoelectric point of BSA is consistent with protein unfolding or denaturation and increased hydrophobicity. In these studies by Davies and Delsignore (1987a), the denaturation of BSA was assessed by solubility in high salt buffer (between pH 4 and 6), where the repulsion of hydrophobic groups by the solvent cage is maximized. In the native (folded) state, hydrophobic residues are shielded from the aqueous environment, but denaturation and unfolding diminish this protection. Since a protein has no net electrical charge at its isoelectric point, the solubilizing properties of hydrophilic residues are minimized. Consequently, a denatured, unfolded protein exhibits decreased solubility at its isoelectric point in high salt buffer because its exposed hydrophobic groups cluster together and cause precipitation. Following exposure of BSA to the hydroxyl radical Davies and colleagues found a 50% loss in soluble BSA as assessed by the method of Bradford (1976), Lowry et al. (1951), and by densitometric analysis following SDS-PAGE of the BSA monomer. Their studies showed that oxygen radicals contributed to BSA denaturation and an increase in hydrophobicity at pH 4. The
denaturation (unfolding) of BSA caused by exposure to the hydroxyl radical was essentially maximal at a molar radical:BSA ratio of 24:1.

The observations of Davies and Delsignore (1987b) outlined above are very similar in theory to the results and observations in the present study with respect to the in vitro NO₂ exposure of α₁-PI and the serum albumins. Exposure of α₁-PI to NO₂ (in N₂) at a molar ratio of NO₂ to α₁-PI of 400:1 resulted in a 50% loss of soluble protein. These exposures were buffered in 0.1 M phosphate pH 7.4, and pH measurements following exposure found no change. However, according to Prutz et al. (1985) gentle bubbling of protein solutions with NO₂/N₂O₄ gas involves rather inhomogeneous conditions and extensive acidification of the system. Based on the observations of Prutz et al. (1985), it is possible that in and around the immediate zone of entry of the NO₂ gas into the α₁-PI solution, an acidic microenvironment may have existed, with temporary changes in pH. Within this acidic microenvironment the α₁-PI molecules may be at or close to their pI of 4.6. NO₂ exposure of α₁-PI led to both precipitation and unfolding, as indicated by increased absorbance at 280 nm, but whether this was due to localized acidification or reaction with NO₂ is not clear. Precipitates of α₁-PI and the serum albumins seem to be a direct result of biphenyl cross-link formation via tyrosine residues, which would enhance hydrophobic clustering among
the side-chain ring structures. The unfolding of the NO$_2$ (in N$_2$) exposed protein as evidenced by its increase in 280 nm absorbance is shown in Figure 13. Nitrogen bubbled control $\alpha_1$-PI which was treated for 32 hours also showed an increase in 280 nm absorbance, but precipitation did not occur. Exposure of HSA to nitrogen for 32 hours resulted in an increase in absorbance at 280 nm with no precipitate whereas exposure of HSA to argon for 32 hours did not result in any increase in absorbance at 280 nm or precipitate. These results indicate that the increase in 280 nm absorbance is not due to NO$_2$ and raised the possibility of secondary reactions involving the nitrogen carrier gas contributing to the increase in 280 nm absorption (unfolding) of $\alpha_1$-PI. Therefore, argon was chosen as an alternative carrier gas in all subsequent exposures with elastin, poly-L-lysine, and the serum albumins.

The Effects of NO$_2$ on Elastin

The release of dimethyllysine residues from the $^3$H-labeled elastin suggested that NO$_2$ was reacting at lysine residues. This was examined further using poly-L-lysine and serum albumins as target molecules. A distinct feature of the insoluble elastin molecule is the presence of lysine derived desmosine cross-links, which are responsible for the insolubility and elasticity of elastin. Virtually all the $^3$H-labeled dimethyllysine residues were released from the insoluble elastin upon exposure to NO$_2$, based on both liquid
scintillation counting and amino acid analysis assays (Table V and Figure 14). Previous studies have shown that lysine is liberated from desmosine upon destruction of the pyridinium ring by mild oxidation with ferricyanide at alkaline pH (Elsden et al. 1963), but the mild reductive methylation conditions used to label the elastin in this work would not have labeled desmosines. Therefore, the released \(^3\)H, which was confirmed by amino acid analysis to be \(^3\)H-dimethyllysine, must have come from lysine residues rather than from desmosines. Evidence for NO\(_2\) mediated fragmentation of polypeptides was provided by gel filtration experiments in which \(^3\)H-poly-L-lysine was degraded into small pieces (Figure 15). These data, along with the amino acid analysis of elastin, indicate that cleavage occurs between amino acid residues in the peptide chain and argue against release of side chain fragments.

The one electron oxidation reduction potential of NO\(_2\) in neutral aqueous solution, \([E(NO_2^-/NO_2^+)]\) is 0.91 V calculated from the N\(_2\)O\(_4\)/2NO\(_2\) couple (Koppenol, 1982), which is somewhat less but comparable to the oxidation reduction potential of ozone (O\(_3\)); \([E(O_3/O_2^-)]\) of 1.6 V. The ozone molecule is a very strong oxidizer and is very similar in shape to the NO\(_2\) molecule. These oxidants also have electron affinities that approximate each other (Peters et al. 1980). The results in the present study suggest that NO\(_2\) and/or its associated nitroxide species preferentially react at lysine-lysine
sequences. Unfortunately, this interpretation is not totally satisfying because bovine elastin does not contain adjacent lysine residues in its sequence (Yeh et al. 1989). While the results with $^3$H-labeled elastin would seem to require cleavage at single lysine residues, cleavage at the more than 50 single lysine residues in the albumins should have resulted in many fragments of various sizes and this clearly was not the case. Thus, the reaction of NO$_2$ with elastin may be unique to that protein, which has a secondary structure that is quite different from that of the albumins. Alternatively, NO$_2$ may cleave at lysines adjacent to other specific residues. Additional studies requiring peptide isolation and amino acid sequencing are needed to answer these questions.

Peptides released by the action of NO$_2$ on elastin or resulting from proteolysis of a more susceptible elastin might lead to cellular responses. Elastin peptides have been shown to be chemotactic for fibroblasts (Senior et al. 1982), alveolar macrophages, and macrophage precursors (Hunninghake et al. 1981). The chemotactic activity associated with elastin derived peptides suggest a mechanism by which alveolar macrophages may accumulate in the lung in NO$_2$ induced "experimental emphysema." Presently there is still much debate concerning the active chemotactic site on the elastin derived peptides. Senior et al. (1982) isolated a 14 to 20 kDa elastin derived peptide with chemotactic
activity that contained a high concentration of desmosine, and purified desmosine cross-links are themselves chemoattractant (Legrand et al. 1989). The direct effects of NO$_2$ on elastin observed in the present study would be expected to, at the very least, result in alterations or modifications in the three-dimensional structure of the elastin molecule, possibly resulting in elastin derived peptides, which may contribute to lung fibrosis and/or attract phagocytes to the lung, which could contribute to inflammation and the pathogenesis of emphysema observed in animals exposed to NO$_2$. Additionally, modification of elastin by NO$_2$ may increase the proteolytic susceptibility of the elastin, as was shown for collagen after exposure to ozone (Curran, 1984).

**Protein Aggregation and Fragmentation**

*as a Result of NO$_2$ Exposure*

In the following discussion the term "protein fragmentation" will be used to refer to the direct breakdown of proteins by oxygen radicals or NO$_2$. Protein fragmentation processes in systems which generate oxygen radicals have been found to involve both main chain scission (Garrison et al. 1962) and side chain scission (Schuessler & Schilling, 1984) and are totally different from peptide hydrolysis. The term protein degradation commonly refers to peptide bond hydrolysis by proteolytic enzymes.
The previously mentioned studies by Davies and Delsignore (1987b) showed that exposure of BSA to OH- generated by irradiation of water by 60Co causes BSA denaturation/increased hydrophobicity, followed by the formation of covalent (bityrosine) bonds between BSA molecules (covalent aggregation). This in vitro exposure of BSA also results in the fragmentation of the BSA monomer to peptides confined to a molecular weight range of 66.2 kDa down to approximately 7 kDa. The authors concluded that the fragmentation occurs predominantly at the α-carbon rather than at peptide bonds. This type of fragmentation occurs as a result of O2 addition to α-carbon radicals induced by OH-. The resulting peroxyl radical is believed to further react to produce a peroxide; and upon decomposition, chain scission occurs, which produces a carbonyl and an amide. In this scenario the peptide bond remains intact. Finally, BSA denaturation by hydroxyl radicals was shown to precede aggregation and fragmentation.

Data presented in the present work shows that exposure of the serum albumins also results in nitration of tyrosine residues, as well as aggregation of the protein structures, and finally in protein fragmentation. Prutz et al. (1985) found that NO2 radicals generated by exposing deaerated nitrite (NO2-) and nitrate (NO3-) solutions to ionizing radiation, results in the formation of nitrotyrosine in the target proteins lysozyme, ribonuclease A, and subtilisin
Carlsburg. Additionally, bityrosine and nitrotyrosine formation was observed when the dipeptide Gly-Tyr was the target molecule. The pulsed radiolysis favored nitration at physiological pH relative to bityrosine formation, but the methods used did not allow detection of reaction with lysines or examine peptide fragmentation. High molecular weight aggregates of $^3$H-BSA and $^3$H-HSA are seen in the present study in Figure 17 (B) (lanes 2 through 6) and Figure 18 (B) (lanes 2 through 5), and treatment of the precipitates from NO$_2$ exposed $^3$H-HSA 36, 48, 72, and 96 hour time points (Figure 19 B lanes 2 through 5) with 1% SDS, and 2% DTT solubilized the samples but failed to disrupt the aggregates. The time course study in Figure 19B revealed that the precipitates predominantly consist of high-molecular weight aggregates of $^3$H-HSA. The failure of SDS and reducing agents to disrupt these aggregates indicates that they result from the formation of biphenolic cross-links of BSA and HSA, in agreement with the findings of Prutz et al. (1985). The detection of bityrosine has also been demonstrated in the high molecular weight, yellow, insoluble protein fraction of human cataractous lens protein (Garcia-Castineras et al. 1978). Davies and Delsignore (1987b) also noted bityrosine formation upon in vitro exposure of BSA to hydroxyl radicals (OH$^-$), and the exposed BSA was found to be more susceptible to proteolysis than native BSA. Additionally increased susceptibility to
proteolysis following O3 exposure has been demonstrated for collagen (Curran et al. 1984) and fibronectin (Vartio et al. 1981). Whether NO2 exposure also increases the susceptibility of proteins to proteolytic degradation has not been addressed, but the possibility seems likely.

The time course study in Figure 19 (B) also confirms the estimated M, of the small NO2 generated peptides of 3H-BSA and 3H-HSA Figures 17 (B) and 18 (B) at a M, = 6,000. This M, is consistent with the proposed M, of the small peptides that would be generated by cleavage at or near the Lys321-Lys521 bond in the sequence of BSA and at or near the Lys521-Lys524 bond in the sequence of HSA. Although BSA and HSA contain additional Lys-Lys sequences, peptides resulting from cleavage at these sites would yield peptides containing tyrosine residues capable of forming aggregates, whereas the C-terminal fragment generated by cleavage at residues 523-524 of HSA would not contain tyrosine and thus could not aggregate via the formation of bityrosine cross-links. This proposed scheme is presented in Figure 20. Evidence for this proposed cleavage site results from our exposure studies with a synthetic polypeptide of lysine residues (3H-Poly-L-Lysine). The gel filtration data show that 3H-poly-L-lysine was extensively fragmented upon exposure to NO2. Collectively, these findings also indicate that NO2 can react with proteins at physiological pH, resulting in the formation of cross-links, the nitration of tyrosine residues
Figure 20. Schematic of proposed cleavage of HSA at lysine-lysine sequences by NO\textsubscript{2} to generate peptides with and without tyrosine residues. These tyrosine residues are essential for the formation of biphenolic aggregates of HSA.
and peptide fragmentation. All of these processes could damage proteins vital to the function of the lung. Modified elastin may not function normally and may be more or less susceptible to proteolysis. Similarly, the function of other lung proteins may be adversely affected by cleavage and/or the formation of cross-links. Determining the mechanism of the lysine cleavage reaction and the extent to which this reaction occurs in vivo will require additional work.

Along with the above mentioned future considerations, it was shown recently that activated macrophages synthesize nitrite (NO$_2^-$), and nitrate (NO$_3^-$) from L-arginine. (Marletta et al. 1988). Nitric oxide (NO') is an intermediate in the synthesis of nitrite, and nitrate in these mammalian cells so that this discovery represents one of the rare instances in mammalian biochemistry where such a reactive species is found as a free intermediate. Prior to this finding exposure to NO$_2^-$ was always thought to result from environmental sources. At the present, the potential biological role of nitric oxide production by these cells is speculative. One possibility consistent with the theme of this discussion is derived from the known cytostasis-inducing and killing function of activated macrophages. This theory as reviewed by Marletta, (1989) suggest that nitric oxide may be acting as a cytotoxic agent, through its chemical properties. This theory is based on a requirement for
arginine for the induction of cytostasis to occur with activated macrophages against various tumor cell lines. The same effect in this system can be reproduced by supplementing these cells with nitric oxide in place of L-arginine. This finding that nitric oxide is an intermediate in the production of nitrite ($\text{NO}_2^-$), and nitrate ($\text{NO}_3^-$), from L-arginine in activated macrophages is very interesting in that this pathway is required for these cells to become bactericidal and/or tumoricidal. Elucidating the mechanisms involved in this pathway may contribute to a better understanding of how the immune system is controlled.


APPENDIX
Amino Acid Analysis of Soluble and Insoluble
NO₂ Exposed ³H-Elastin Fractions and ³H-Poly-L-Lysine.

The total CPM/5 mg in 5 ml of control insoluble ³H-
Elastin = 90,875 CPM/5 ml = 18,165 CPM/ml.

Following exposure of the ³H-Elastin samples to NO₂ all
samples were centrifuged at 10,000 x g for 30 minutes. The
total radioactivity associated with the soluble
(supernatant) fractions was determined by sampling 500 µl of
these fractions in 10 ml of Cytoscient liquid scintillation
cocktail. The remaining supernatants (4.5 ml) were removed
with a pasteur pipette, the insoluble fractions were
resuspended in 3.0 ml of 0.1 M sodium phosphate exposure
buffer pH 7.4, and 200 µl of each resuspended insoluble ³H-
elastin fraction was counted in 5 ml of liquid scintillation
cocktail to determine CPM/ml.

(A) 500 pmoles of Amino Acid Standard H.

(B) Control Insoluble ³H-Elastin (31,345 CPM/ml). Thirty
microliters (30 µl) was hydrolyzed as described in Materials
and Methods and resuspended in 400 µl diluent. Fifteen
microliters (15 µl) was analyzed which gave 0.546 nmoles of
dimethylylsine (DMK) and 0.546 nmoles DMK x (128 ng/nmole)=
69.8 ng DMK/15 µl analyzed. The total DMK/400 µl diluent=
1,884 ng= 1.8 µg/ 30 µl hydrolyzed. This equals 62.04 µg/ml
x 1.78 ml = 113.8 µg TOTAL DMK.

(C) 9 hr. NO₂ Exposed Insoluble ³H-Elastin (15 pmoles
NO₂); (27,658 CPM/ml). Forty microliters (40 µl) was
hydrolyzed as described in Materials and Methods and
resuspended in 200 µl diluent. Ten microliters (10 µl) was
analyzed which gave 0.553 nmoles of DMK and 0.553 nmoles DMK
x (128 ng/nmole)= 70.78 ng/10 µl analyzed. The total
DMK/200 µl diluent= 1,415ng= 1.4 µg/ 40 µl hydrolyzed. This
equals 32.25 µg/ml x 2.7 ml = 95.17 µg TOTAL DMK.

(D) 18 hr. NO₂ Exposed Insoluble ³H-Elastin (30 pmoles
NO₂); (15,440 CPM/ml). Seventy microliters (70 µl) was
hydrolyzed as described under Materials and Methods and
resuspended in 200 µl diluent. Ten microliters (10 µl) was
analyzed which gave 0.576 nmoles DMK and 0.576 nmoles DMK x
(128 ng/nmole)= 73.72 ng/10 µl analyzed. The total DMK/200
µl diluent= 1.474ng= 1.47 µg/ 70 µl hydrolyzed. This equals
21.02 µg/ml x 2.7 ml = 56.75 µg TOTAL DMK.
(E) 36 hr. NO₂ Exposed Insoluble ³H-Elastin (60 μmoles NO₂); (12,420 CPM/ml). Eighty-four microliters (84 μl) was hydrolyzed as described under Materials and Methods and resuspended in 200 μl diluent. Eight microliters (8 μl) was analyzed which gave 0.534 nmoles DMK and 0.534 nmoles DMK x (128 ng/nmole) = 68.35 ng/8 μl analyzed. The total DMK/200 μl diluent = 1,708 ng = 1.7 μg/84 μl hydrolyzed. This equals 20.23 μg/ml x 2.7 ml = 54.62 μg TOTAL DMK.

(F) 72 hr. NO₂ Exposed Insoluble ³H-Elastin (120 μmoles NO₂); (6,495 CPM/ml). One-Hundred sixty microliters (160 μl) was hydrolyzed as described under Materials and Methods and resuspended in 200 μl diluent. Eight microliters (8 μl) was analyzed which gave 0.211 nmoles DMK and 0.211 nmoles DMK x (128 ng/nmole) = 27 ng/8 μl analyzed. The total DMK/200 μl diluent = 675 ng = 0.675 μg/160 μl hydrolyzed. This equals 4.2 μg x 2.7 ml = 11.34 μg TOTAL DMK.

(B') Control Soluble ³H-Elastin (1,104 CPM/ml). Two Hundred microliters (200 μl) was hydrolyzed as described in Materials and Methods and resuspended in 200 μl diluent. Ten microliters (10 μl) was analyzed which gave 0.119 nmoles DMK and 0.119 nmoles DMK x (128 ng/nmole) = 15.28 ng/10 μl analyzed. The total DMK/200 μl diluent = 304.6 ng = 0.304 μg/200 μl hydrolyzed. This equals 1.52 μg x 5 ml = 7.6 μg TOTAL DMK.

(C') 9 hr. NO Exposed Soluble ³H-Elastin (6,258 CPM/ml). One Hundred-Sixty microliters (160 μl) was hydrolyzed as described under Materials and Methods and resuspended in 200 μl diluent. Ten microliters (10 μl) was analyzed which gave 0.234 nmoles DMK and 0.234 nmoles DMK x (128 ng/nmole) = 29.95 ng/10 μl analyzed. The total DMK/200 μl diluent = 599 ng = 0.599 μg/160 μl hydrolyzed. This equals 3.78 μg x 5 ml = 18.9 μg TOTAL DMK.

(D') 18 hr. NO₂ Exposed Soluble ³H-Elastin (6,760 CPM/ml). One Hundred-Fourty Five microliters (145 μl) was hydrolyzed as described under Materials and Methods and resuspended in 200 μl diluent. Ten microliters (10 μl) was analyzed which gave 0.319 nmoles DMK and 0.319 nmoles DMK x (128 ng/nmole) = 40.83 ng/10 μl analyzed. The total DMK/200 μl diluent = 816.6 μg/145 μl hydrolyzed. This equals 5.71 μg x 5 ml = 28.5 μg TOTAL DMK.

(E') 36 hr. NO₂ Exposed Soluble ³H-Elastin (10,108 CPM/ml). One Hundred microliters (100 μl) was hydrolyzed as described under Materials and Methods and resuspended in 200 μl diluent. Ten microliters (100 μl) was analyzed which gave 0.25 nmoles DMK and 0.25 nmoles DMK x (128 ng/nmole) = 32 ng/10 μl analyzed. The total DMK/200 μl diluent = 6.4 μg x 5 ml = 32 μg TOTAL DMK.
(F') 72 hr. NO₂ Exposed Soluble ³H-Elastin (16,394 CPM/ml). Sixty-Five microliters (65 μl) was hydrolyzed as described under Materials and Methods and resuspended in 200 μl diluent. Eight microliters (8 μl) was analyzed which gave 0.365 nmoles DMK and 0.365 nmoles DMK x (128 ng/nmole) = 46.7 ng/8 μl analyzed. The total DMK/200 μl diluent = 17.98 μg x 5 ml = 90 μg TOTAL DMK.

(G) Argon Bubbled Control ³H-Poly-L-Lysine. Ten microliters (10 μl of 1 ml), (803 CPM of 8.0 x 10³ CPM) was analyzed and 98% of the injected radioactivity was recovered in fractions eluting between 17 and 18 minutes which gave 0.4 nmoles DMK. The total DMK/200 μl diluent = 8 nmoles = 1.024 μg TOTAL DMK.

(G') 72 hr. NO₂ Exposed ³H-Poly-L-Lysine. Ten microliters (10 μl of 1 ml), (2,402 CPM of 2.4 x 10⁴ CPM) was analyzed and 90% of the injected radioactivity was recovered in fractions eluting between 16.5 and 17.5 minutes which gave 1.1 nmoles of DMK. The total DMK/200 μl diluent = 22 nmoles= 2.816 μg TOTAL DMK.

**AMINO ACID STANDARD**
INSOLUBLE $^3$H-ELASTIN

B
CONTROL

C
9 hr

K
SUPERNATANT 3H-ELASTIN

CONTROL

9 hr
$F^9$

72 hr
72 HR

CONTROL

3H-DMK

3H-POLY-L-LYSINE
# VITA

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